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# *SOME MECHANISMS OF CONSCIOUSNESS DISCOVERED DURING ELECTRICAL STIMULATION OF THE BRAIN**

By Wilder Penfield

MONTREAL NEUROLOGICAL INSTITUTE, MONTREAL, CANADA

*Read before the Academy, November 18, 1957*

This great National Academy embraces many sciences—almost as many, in fact, as there are members—for this is the day of diversity of thought and specialization of technique. But, although there are many sciences, there is only one scientific method and only one brain. Surely, there is no problem in any field more vast than the problem of the mechanisms of the brain and its relation to the mind. In these unstable times scientists would do well to give some thought to the nature of man himself.

The first function of a physician is to cure men and women of their diseases and to comfort when he cannot cure. But that is not enough. He must help to create a true science of the body and the mind of man. It was a physician who first introduced the scientific method into the study of nature. That was in Greece in the fifth century before Christ, when Hippocrates, an Asclepiad of Cos, opposed the philosophers who strove to explain all natural phenomena by "unprovable hypotheses." Indeed, the word "hypothesis" is said to make its first appearance in his writings. Observe and record the ways of nature, he told his disciples. Then conclude as best you can, for "life is short, the art long, opportunity fugitive, experience deceptive, judgment difficult."[1]

It was evidently as the result of study of epileptic patients that Hippocrates drew his own conclusions about the brain. For it was in his lecture on the scared disease—the Greek term for epilepsy—that he turned away from the current conception of the heart as the organ of reason. Listen to his words: "Men ought to know," he said, "that from the brain and from the brain alone, arise our pleasures, joys, laughter and jests, as well as our sorrows, pains, griefs and tears. Through it, in particular, we think, see, hear—and distinguish the ugly from the beautiful, the bad from the good, the pleasant from the unpleasant." Perhaps this summarizes well enough the knowledge of the human brain for the average man today. In Figure 1 you see the human brain removed from the skull and stripped of its covering membranes. If Hippocrates were here tonight, what could I tell him about the mechanisms within this master organ?

For our recent gains in knowledge we owe a debt of gratitude to workers in the physical sciences. Nevertheless, in this talk I shall assume that no one here has any knowledge of the brain more recent than the statements of Hippocrates.

I shall use terms so simple that they will be comprehensible even to physicists whose minds have slipped away, bemused, into outer space.

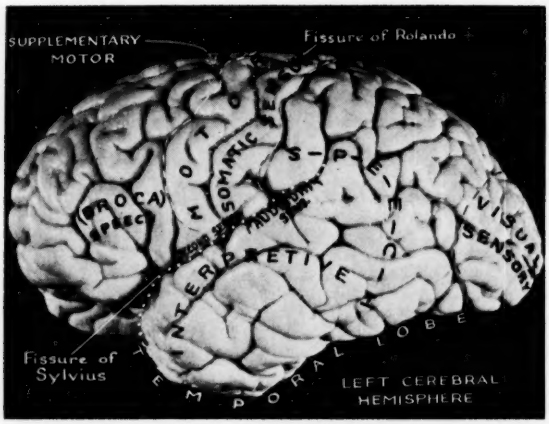


FIG. 1.—Human brain

As a neurosurgeon, it has been my lot to watch the experiments which only disease and injury can carry out upon the brains of unfortunate men and women. Of recent years my special province has been the surgical treatment of epilepsy, removing abnormal areas of the brain, areas in which the unbridled electrical discharges arise that produce epileptic seizures. There are many brain areas that can be removed with little or no detectable functional loss.

During such surgical procedures, the skull is opened and the brain exposed under local anesthesia, while the patient lies on the operating table fully conscious. Only thus is the cause of the attack to be found, and the surgeon's hand guided. The patient talks and answers the surgeon's questions while he maps out the various functional areas by applying a gentle electrical stimulus here and there on the cortex. The pattern of fissures and convolutions which you see in Figure 1 is never twice the same. The electrode is needed for orientation.

Sir Charles Sherrington,[2] in whose laboratory I was once a graduate student of physiology, said that brain function is made possible by "transient electrical potentials traveling the fibers of the nervous system."

The transient potentials travel along these fibers from the sense organs to the brain—from the eye and ear, joints, muscles, nose, and skin to the brain. And from the brain, potentials travel outward along other nerve fibers to the muscles to cause them to move in voluntary action. This seems clear enough except that Hippocrates would not have understood the meaning of "potential" or of "electricity." I must confess that I don't either. Here we would have had something in common.

Our present problem is to single out within the total function one small mechanism after another. There was little valid evidence of any localization of function within the nervous system, in spite of the amusing claims of the phrenologists, until Paul Broca,[3] a French surgeon, proved by autopsy in 1861 that a small area

of destruction in an otherwise normal brain had produced loss of the ability to speak without loss of other abilities. The lesion he described was in the third convolution of the frontal lobe of the left hemisphere (Fig. 1). This is the so-called dominant hemisphere for a right-handed man.

After Broca, the next great advance was the demonstration by Fritsch and Hitzig,[4] in 1870, that an electric current, applied by an electrode placed on the cerebral cortex of a lightly anesthetized dog, caused one leg alone to move on the opposite side of the body. Fritsch and Hitzig reasoned hopefully that they had found the very place where spirit and body could meet. This, they wrote, was "the place of entry of single psychic functions into material."

Then it was that the neurophysiologists, led by Ferrier, came crowding into the newly opened field of brain localization. During the past century they have had their heyday. With the recent help of electronic engineers and neuroanatomists, their barns are bursting now with new-found facts. The meaning of these facts is not so simple. Much grain remains to be threshed.

There is a visual, auditory, and somatic area for sensation in the cortex of each side. There is another area on each side that is clearly motor, as Fritsch and Hitzig pointed out. This motor area has to do with voluntary, rather than reflex, movement.

The experimentalists, led by Sherrington in one school and Pavlov[5] in another, have come to understand many of the mechanisms of subconscious reflex action, by their study of laboratory animals.

The cerebral hemispheres are covered by a gray carpet of millions of nerve cells, the cerebral cortex. In the central portion of the brain is the brain stem, made up of gray matter and interconnecting nerve fibers.

The integrative activity that comes between sensory input and motor output must obviously depend in large part upon the connections of the cortex with the underlying brain stem. The central or centrencephalic organization of electrical potentials constitutes the physical basis of the mind. But the detail of its action remains a mystery. To approach this mystery we must study man himself.

Electrical stimulation of the cerebral cortex of conscious men has verified and amplified the mapping of motor and sensory areas of the cerebral cortex as worked out in laboratory mammals. But it has done something else; and that forms the subject of this address.

*Experiential Responses to Stimulation.*—Twenty-six years ago I was operating upon a woman under local anesthesia in the Royal Victoria Hospital and was applying to different points on the temporal lobe of her brain a stimulating electrode. She (E. W.) told me suddenly that she seemed to be living over again a previous experience. She seemed to see herself giving birth to her baby girl. That had happened years before, and meanwhile the girl had grown up. The mother was now lying on the operating table in my operating room, hoping that I could cure her attacks of focal epilepsy.

This, I thought, was a strange moment for her to talk of that previous experience, but then, I reflected, women were unpredictable, and it was never intended that men should understand them completely. Nevertheless, I noted the fact that it was while my stimulating electrode was applied to the left temporal lobe that this woman had had this unrelated and vivid recollection. That was in 1931.

It was more than five years later when a somewhat similar psychical state made its appearance during electrical stimulation. This time, however, it seemed certain that the stimulus had somehow summoned a past experience.

The Montreal Neurological Institute was opened in 1934, and a patient, J. V., a girl of fourteen years, was admitted there in June, 1936.[6] She was complaining of seizures during which she sometimes fell unconscious to the ground in an epileptic convulsion. But, immediately preceding such an episode, she was aware of what seemed to be a hallucination. It was always the same, an experience came to her from childhood.

The original experience was as follows: She was walking through a meadow. Her brothers had run on ahead along the path before her. A man following her said to her that he had snakes in the bag he was carrying. And she was frightened and ran after the brothers. This had been a true experience. Her brothers remembered, and her mother remembered hearing of it.

Afterward, for some years, the experience came back to her in her sleep, and she was said to have a nightmare. Finally, it was recognized that this little dream was a preliminary to an epileptic seizure that might come on at any time, day or night. And the dream sometimes constituted all there was of the attack.

At operation, under local anesthesia, I mapped out the somatic sensory and motor areas for purposes of orientation, and I applied the stimulator to the temporal cortex. "Wait a minute," she said, "and I will tell you." I removed the electrode from the cortex. After a pause, she said: "I saw someone coming toward me, as though he was going to hit me." It was obvious also that she was suddenly frightened.

Stimulation at a point farther forward caused her to say, "I imagine I hear a lot of people shouting at me." Three times, at intervals and without her knowledge, this second point was stimulated again. Each time she broke off our conversation, hearing the voices of her brothers and her mother. And on each occasion she was frightened. She did not remember hearing these voices in any of her epileptic attacks.

Thus the stimulating electrode had recalled the familiar experience that ushered in each of her habitual attacks. But stimulation at other points had recalled to her other experiences of the past, and it had also produced the emotion of fear. Our astonishment was great, for we had produced phenomena that were neither motor nor sensory, and yet the responses seemed to be physiological, not epileptic.[7]

We have recorded many examples of psychical responses to stimulation in recent years. Let me illustrate:

Case D. F.[8] was a young woman of twenty-six years, who worked as an office secretary. She had epileptic attacks, which were ushered in by a warning feeling of sudden fear. The cause was abnormality of the right temporal lobe due to asphyxia in childhood.

Operation was carried out, under local anesthesia, in the right temporal region. I shall describe the steps as simply as possible: An incision was made in the scalp, as outlined in Figure 2. The bone was cut and turned down with its attachment to the scalp, to be replaced and fastened in position after operation. The brain was exposed, as shown in Figure 3.

A gentle electrical current was then applied at one point after another. Those

FIG. 2.—Patient, D. F., on operating table

FIG. 3.—Case D. F. Right hemisphere exposed by osteoplastic craniotomy under local anesthesia. Arrow points to cortical abnormality which was more marked on the mesial surface. Numbered tickets indicate points at which electrical stimulation produced positive responses.

points at which stimulation produced some effect were marked by dropping a numbered ticket on the cortex, as shown in Figures 3 and 4. The nature of the response was dictated by the surgeon to a secretary who sat behind glass in the viewing stand. Bodily sensations were produced, in part, as follows:

*3.* "Tingling" in the left thumb.
*10.* A "jumping" sensation in the lower lip on the left side.
*15.* Numbness in tongue.

Motor responses occurred, in part, as follows:

*13.* Twitching of left side of face and slight protrusion of tongue.
*11.* The patient opened her mouth and vocalized with a steady vowel sound: A—A—A until the electrode was withdrawn.

It was now clear that we had located the central fissure of Rolando, as indicated by the line of white dots, with motor gyrus in front of it and the somatic sensory gyrus behind.

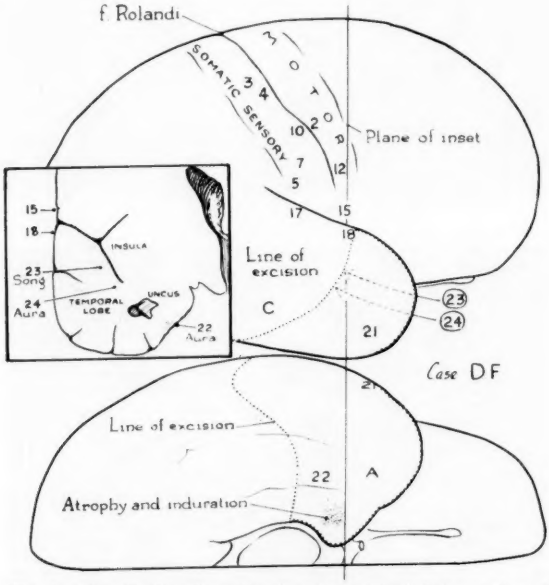


Fig. 4.—Case D. F. Operative drawing. The inferior surface of temporal lobe is shown below and a cross-section in the inset.

Next, an attempt was made to produce the warning of the patient's attack by stimulating different points on the temporal lobe below the fissure of Sylvius, which is marked by the horizontal line of white dots. This failed, until the temporal lobe was elevated and the electrode applied at point *22*, as shown in Figure 4. Then she felt the warning that she had called fear, "the one," she said, "I get before an attack."

This, together with the abnormal spontaneous electrical activity that my associate Dr. Herbert Jasper, reported in his electrograph when I placed a recording electrode at point *A* not far off, made it obvious that the general origin of the seizures had been located. And so the anterior end of the temporal lobe was removed along the dotted line, as shown in Figure 4. A vertical cross-section of the temporal lobe, in the line of removal, is shown in the inset.

When the electrode was applied in gray matter on the cut face of the temporal lobe at point *23*, the patient observed: "I hear some music." Fifteen minutes later, the electrode was applied to the same spot again without her knowledge. "I hear music again," she said. "It is like radio." Again and again, then, the electrode tip was applied to this point. Each time, she heard an orchestra playing the same piece of music. It apparently began at the same point and went on from verse to chorus. Seeing the electrical stimulator box, from where she lay under the surgical coverings, she thought it was a gramophone that someone was turning on from time to time.

She was asked to describe the music. When the electrode was applied again, she began to hum a tune, and all in the operating room listened in astonished silence. She was obviously humming along with the orchestra at about the tempo that would be expected.

Other points were stimulated with no result, except at three points, quite close to *23*, where the same song was reproduced.

After the patient had returned home, she wrote to me on April 16, 1950. The letter was, in part, as follows:

"Today is a year that you operated on me, and I suppose you are wondering how I am coming along. . . .

"Now to answer your questions: I heard the song right from the beginning, and you know I could remember much more of it right in the operating room. . . .

"There were instruments. . . . It was as though it were being played by an orchestra. Definitely it *was not* as though I were imagining the tune to myself. I actually heard it. It is not one of my favorite songs, so I don't know why I heard that song. I finally got ahold of a copy of this piece and played it on the piano the other Sunday.

"Thanks again for better health."

In other patients, there have been other similar examples of music production. Stimulation of the surface of the temporal cortex on one side or the other has caused the patient to hear an organ, a complete orchestration, a voice, or a piano. One patient heard the music and saw the man that was playing it at a piano. A boy reported men sitting in chairs singing. Always it was the reproduction of a previous experience. The tempo of the music was neither faster nor slower than was to be expected.

A young woman (N. C.) said, when her left temporal lobe was stimulated anteriorly, at point *19* in Figure 5, "I had a dream, I had a book under my arm. I was talking to a man. The man was trying to reassure me not to worry about the book." At a point 1 cm. distant, stimulation at point *20* caused her to say: "Mother is talking to me." Fifteen minutes later the same point was stimulated: The patient laughed aloud while the electrode was held in place. After withdrawl of the electrode, she was asked to explain. "Well," she said, "it is kind of a long story, but I will tell you. . . ."

After an interval of time, the electrode was applied again, without warning, at point *20*. The patient spoke quietly while the electrode was kept in place: "Yes, another experience," she said. "A different experience, a true experience. This man, Mr. Meerburger, he—oh well, he drinks," etc. Stimulation at *23* caused her to hear music.

One must conclude that there is, hidden away in the brain, a record of the stream of consciousness. It seems to hold the detail of that stream as laid down during each man's waking conscious hours. Contained in this record are all those things of which the individual was once aware—such detail as a man might hope to remember for a few seconds or minutes afterward but which are largely lost to voluntary recall after that time. The things that he ignored are absent from the record.

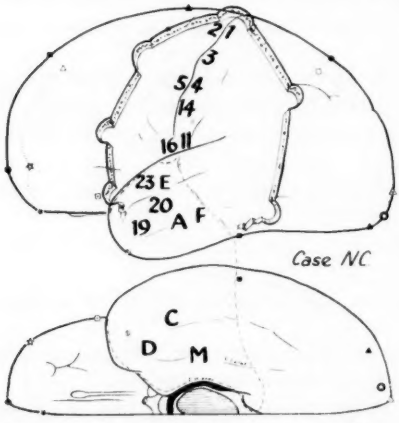


FIG. 5.—Case N. C. Drawing of operation. Numbers indicate the positive responses to stimulation. Letters indicate sites of spontaneous epileptic electrical abnormality determined by electrocorticography.

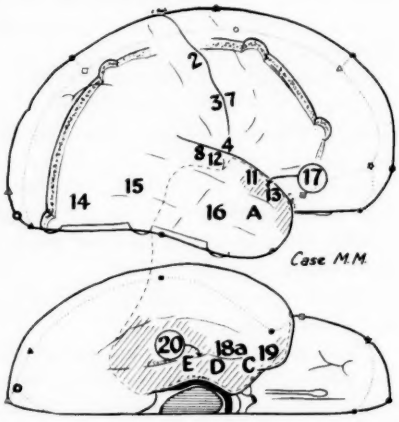


Fig. 6—Case M. M. Drawing at operation. The shaded area indicates abnormal area of cortex, considered epileptogenic. Operative removal carried out along the broken line.

Years ago, William James (1910)[9] described the "stream of consciousness" as a river forever flowing. Its content, he pointed out, was never the same from moment to moment. The record of this stream, as we have brought it to light with the stimulating electrode, might better be compared to the sequence on a wire recorder or to a continuous filmstrip with sound track.[10]

As compared with motor and sensory responses, these might be called psychical or experiential. The psychical responses may be produced by threshold stimulation without ensuing afterdischarge. They have never been produced by stimulation of the frontal or occipital lobes, nor in the central area. One must assume that the epileptic state tends to sensitize the temporal cortex so that it will respond more easily in a positive manner to the stimulating electrode. When a source of epileptic discharges is near the sensory or motor areas of the cortex, they too, become more easily stimulable. The experiences reproduced are usually unimportant ones. They had often been "forgotten," although the subject never seemed to doubt that they were his own experiences. In the great majority of cases, these experiences formed no part of any previous epileptic attack.

The experience that appears as the first experiential response when stimulation is begun in any case seems to depend on chance. It may be recent or it may come from childhood many years before. When a response has been produced, however, it seems to have an immediately facilitating effect on the result of subsequent stimulations. A second stimulus at approximately the same point, if not too long

delayed, is apt to reproduce the same experience, beginning at the same moment of time. This was true, you remember, in the case of D. F. described above. She heard the playing of a certain song, the same song, each time the electrode was applied.

If restimulation of the same point does not reactivate the same strip of time, it is apt to produce an experience that is similar in content or subject. For instance, a boy, R. W., heard his mother talking on the telephone when two points 3 cm. apart were stimulated twice in quick succession. After a lapse of time when the electrode was set down between the two points, he heard his mother talking with his brother. The similarity of the experience produced does suggest a greater availability of experiences that are related to one another by some common characteristic.

This is borne out by the results of stimulation in the case of M. M. She heard "a mother calling her little boy" when point *11* on the first temporal convolution was stimulated (Fig. 6). When it was repeated at once, without warning, she heard the same thing. When repeated again twice at the same point, she heard it each time, and she recognized that she was near her childhood home.

At point *12* nearby, on the same convolution, stimulation caused her to hear a man's voice and a woman's voice "down along the river somewhere." And she saw the river. It was at a place "I was visiting," she said, "when I was a child."

Three minutes later, while the electrode was held in place at *13*, she exclaimed that she heard voices late at night and that she saw the "big wagons they used to haul the animals [of a circus] in."

Eleven minutes later, the original point, *11*, was stimulated again. She no longer heard the mother calling her little boy. Instead, she heard "the voices of people calling from building to building."

Later still, when a coated electrode was inserted at *17* so as to stimulate the first temporal convolution deep in the fissure of Sylvius, she said, "I had. . .a familiar memory, in an office somewhere. I could see the desks. I was there, and someone was calling to me, a man leaning on a desk with a pencil in his hand."

The time had changed and the scene. It was at least 10 years later, evidently after she had become a stenographer, and yet the calling was common to all the experiences.

*Discussion of Experiential Responses.*—Curiously enough, two experiences or strips of time are never activated concurrently. Consequently, there is no confusion. There seems to be an all-or-nothing organization which inhibits other records from being activated.

This is not a memory, as we usually use the word, although it may have some relation to it. No man can recall by voluntary effort such a wealth of detail. A man may learn a song so that he can sing it perfectly, but he probably cannot recall in detail any one of the many times he heard it. Most things that a man is able to recall to memory are generalizations and summaries. If it were not so, we might find ourselves confused, perhaps, by too great a richness of detail.

Many a patient has told me that the experience brought back by the electrode is much more real than remembering. And yet he is still aware of the present situation. There is a doubling of consciousness, and yet he knows which is the present. He may cry out in astonishment that he is hearing and seeing friends

who he knows are far away. J. T., realizing that he was on the operating table in Montreal, cried out: "Yes, doctor, yes, doctor! Now, I hear people laughing—my friends in South Africa." It seemed to him that he was laughing with them.

In general, it may be said that if the experience of that previous time, now so strangely under review, was accompanied with fear or amusement or admiration, he has those same reactions again.

The responses of these patients have been verified and checked in every way possible. Unexpected statements are proved by restimulation without warning and sometimes by warning without stimulation, before they are accepted. The brain has no sensation of its own. Consequently, the patient has no possible means of knowing when the electrode is applied, unless he is told or unless he is aware of a positive physiological effect.

*The Ganglionic Record.*—How is this record of the past stored in the brain? and where? One may assume that at the time of the original experience electrical potentials passed through the nerve cells and nerve connections of a recording mechanism in a specific patterned sequence and that some form of permanent facilitation preserves that sequence so that the record can be replayed at a later time, in a manner analogous to the replaying of a wire recorder or tape recorder. But this remains a supposition.

*Reconsideration of Stimulation Responses.*—In the Sherrington Lecture delivered in Liverpool last January,[12] I reviewed all our records of stimulation of the human cerebral cortex, to see whether I could answer these questions of how and where. The number of such patients operated upon under local anesthesia was then just short of a thousand.

Many stimulations—the majority, in fact—are greeted by silence. Not all areas of the brain can respond, because of the nature of their function. The normally responsive areas are often in a state that seems to block translation of electrical pulse into physiological activity. There are many things that stimulation can never produce. No constructive thinking is produced, no willed or purposeful behavior. In general, application of this crude electrical current seems to interfere with the normal functional employment of the cortex itself in the area to which the electrode is applied.

For example, if it is applied to one of the speech areas of the dominant hemisphere, such as Broca's convolution, the patient is silent. No words can be produced that way. If the patient is asked to answer while the electrode is still applied, he discovers, to his surprise, that he is aphasic. That is, he can no longer find words to express his thought. But they come with a rush when the electrode is lifted and he says, then, the things he was trying to say while the electrode was interfering with his employment of the speech area of cortex.

My conclusion is that the electrode can produce a positive physiological effect only in those areas of cortex that normally send currents of neuronal impulses to distant collections of nerve cells to activate mechanisms there. Apparently the local electrical state of the cortex must be converted into a physiological corticofugal conduction of electrical potentials.

If an electrical stimulus of 60 pulses per second, for example, is applied to his visual sensory cortex (Fig. 7), the effective stream of neurone impulses passes centrally, not back to the eye. It passes into the subcortical vision-receiving center

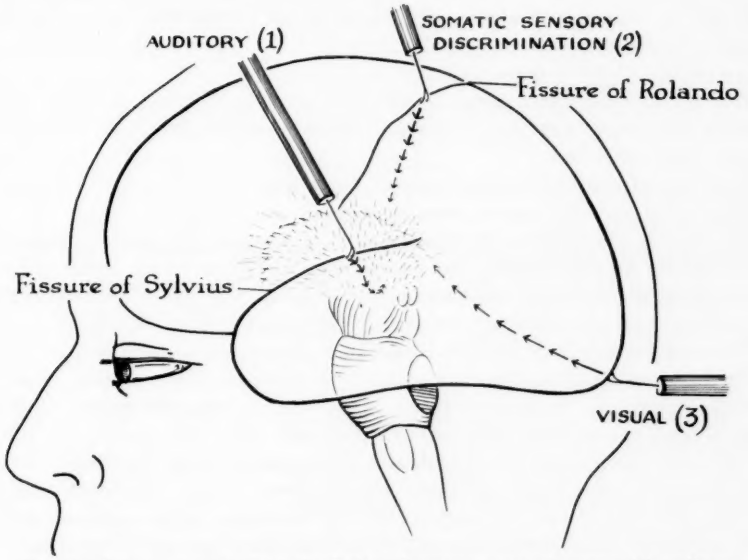


FIG. 7.—Sensory responses to stimulation of sensory areas of cortex: (*1*) auditory, (*2*) somatic sensory, (*3*) visual. Electrical stimulation produces sensation by means of dromic neuronal conductions from each cortical area to a corresponding subcortical receiving center.

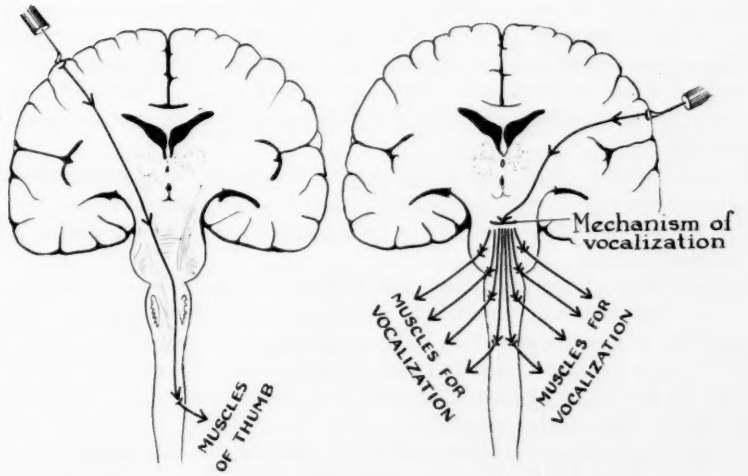


FIG. 8.—Motor responses to stimulation of the pre-Rolandic motor convolution produced by corticofugal neuronal conduction to *A*, anterior horn cells of spinal cord, and *B*, vocalization and respiratory mechanism of brain stem.

because the visual cortex normally serves as a way-station in the pathway of normal potentials that leads from eye through the cortex and on into the centrencephalic circuits of organization in the brain stem.

Exactly the same current applied to the auditory area of cortex, or to the somatic sensory area, sends a stream of neurone impulses inward (Fig. 7), and the patient seems to hear or to feel.

This flow of neuronal pulses produces crude sensations of vision or hearing or tactile sensation. No man is seen, nor is any voice heard to speak. If more clever stimulators could be constructed to vary the time and place of stimulation in a properly planned pattern, the patient might be made to see a man or hear a voice, perhaps. But there would be no sense of familiarity. It would be a synthetic man and a synthetic voice, like the products of the art of Walt Disney.

When the stimulating electrode is applied to the motor convolution just in front of the fissure of Rolando, there are two types of response, one gross and simple, the other complicated. In the first instance (Fig. 8, *A*), a stream of neuronal impulses passes down through the corticospinal tract and out to the peripheral muscles. If the point touched was the thumb area of the motor gyrus, the thumb flexes or extends and remains in that position. He is helpless to resist. He cannot use that thumb, although he can reach across and seize the thumb with the other hand.

If, however, the electrode was placed on that small portion of the motor convolution that is devoted to vocalization (Fig. 8, *B*), the patient, quite helplessly, carries out a very complicated movement—opening his mouth, contracting his diaphragm, and uttering a vowel sound—*A*—*A*—*A* until his breath is gone. He then stops, and, after taking a breath, he continues. In this case the stream of neuronal impulses passes directly to a vocalization and respiratory mechanism in the brain stem. There is nothing one-sided about the movement of this mechanism. It can also be activated by stimulation in the supplementary motor area of the same hemisphere and at the two homologous points in the opposite hemisphere.

Thus a complicated performance can be produced by an electrode on the cortex, as well as simple performances. But the stream of neuronal potentials must be caused to pass away from the area of stimulus along a route of normal neuronal conduction.

It seems evident, then, that in the case of sensory and motor stimulation the conduction must be dromic, that is, in the direction of normal functional flow. If this is accepted tentatively as the rule governing such reactions, we may return to the temporal cortex.

I would conclude here, too, that when activation of the stream of previous consciousness is produced by stimulation, there is conduction away from the neighborhood of the stimulating electrode. The ganglionic record of past experience must therefore lie at a distance from that area of temporal cortex. But the key that can unlock the past is to be found there and apparently only there. The following observations bear out this suggestion.

*Interpretive Responses to Stimulation.*—In addition to the experiential flashbacks (A in Table 1), which we have been discussing, there is one other type of response. This response may follow stimulation of the same general area of temporal cortex and only there. When the electrode is applied, the patient has a sudden "feeling" about the present situation (B in Table 1). It is an interpretation

of the present, but not one that the patient thinks out deliberately. It is a signal, for example, that the present situation is familiar, that it has been experienced before. Or it is strange, perhaps. It may be a signal that things seen are growing larger or sounds heard are louder, a signal that something is approaching. Or it may be the opposite—things going away. It may be a signal of change in the erectness of things or in perspective. It may be a sudden feeling of fear, an interpretation that the present situation is dangerous.

TABLE 1

PSYCHICAL RESPONSES TO ELECTRICAL STIMULATION OF INTERPRETATIVE AREAS OF CORTEX

A. *Experiential Flash-back:* Random re-enactment of a conscious sequence from the patient's past.
B. *Interpretive Signaling:* Production of sudden interpretations of the present experience, such as familiar, strange, fearful, coming nearer, going away, etc.

TABLE 2

INTERPRETIVE ILLUSIONS: ALTERATIONS OF PERCEPTION OF THE PRESENT

1. Auditory Illusions
   Distance, Loudness, Tempo
2. Visual Illusions
   Distance, Dimension, Erectness, Tempo
3. Illusions of Comparison
   Familiarity, Strangeness, Unreality
4. Illusional Emotions
   Fear, Loneliness, Separation, Sorrow, Disgust

For example, the patient M. M. (Fig. 6) said, when point *14* on the posterior limit of the right temporal lobe was stimulated, "The whole operation now seems familiar." The "feeling" passed when the electrode was lifted. She was then told that the stimulation would be repeated, but no stimulus was applied. "Nothing," she said. Then point *15* was stimulated, and she said, "Just a tiny flash of familiarity and a feeling that I knew everything that was going to happen in the near future." To explain this, she added, "as though I had been through all this before."

When these interpretations of the present are produced by the electrode, we call them illusions, since they are false interpretations[13] (see Table 2). But in normal life and under normal conditions these feelings are not illusions. They are true. They are reliable signals that can rise into consciousness only after a comparison is made between past records and the present experience. How else could the sudden awareness that this has happened before come to us with true meaning? Or how else could we know that this or that brings danger before we have had "time to think"?

*Cortex for Comparative Interpretation.*—Thus it is evident that the temporal cortex, on stimulation (Fig. 9), yields two types of response which are psychical rather than sensory or motor. The two forms are (1) a flash-back of past experience and (2) a signaling of interpretation of the present experience. The two types of response would seem to form parts of one subconscious process, the process of comparing present experience with past similar experiences.

The area of cortex from which these responses are obtained in both hemispheres is one to which no function has been assigned. It covers most of the superior surfaces

of the temporal lobes, as well as the lateral and probably the inferior surfaces. For the purposes of further study this area of the cortex might be given a name to distinguish it from motor and sensory areas and the speech areas. This seems to be the area for *comparative interpretation* and might be called that or, more briefly, the *interpretive cortex* (see Fig. 1).

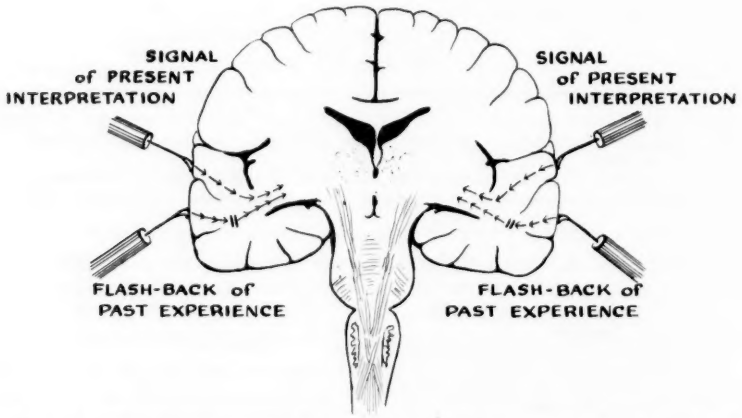


FIG. 9.—"Psychical" responses to stimulation of the (comparative) "interpretive" cortex of temporal lobe. The lines of arrows indicate the hypothetical pathways of neuronal impulses leading into the circuits of centrencephalic integration. In the case of the "flash-back" pathway, the interruption by vertical lines represents the ganglionic record of the past without attempting to indicate its position.

I surmise that the interpretive cortex makes its normal functional contribution in situations such as the following:

When you meet an acquaintance of years gone by, whom you might have thought forgotten, you may be startled first by a sudden signal of familiarity, because of the sound of his voice, his smile, his way of walking. Almost instanteously some strange mechanism of the brain is providing you with a standard of comparison. You see how this present man differs from the acquaintance in the past—the man you have not thought of for many years. A moment earlier you could not have pictured him. Now you can compare the past with the present in great detail. You detect the slightest change in face or hair. You note that his movements are slowed, the hair thinned, alas! the shoulder stooped. But his laugh, perhaps, has not changed.

I would assume that the comparative-interpretive cortex of the temporal lobes has somehow managed the selection and activation of the short strips of past conscious experience in which this man was once the focus of your attention. It makes possible the scanning process by which past experiences, however scattered they may have been in time, are selected and made available to the present, for the purpose of comparative interpretation.

The problem now is to understand how these functional areas are employed in the total integration of the traveling potentials. That integration constitutes the neural basis of consciousness. The problem is to work out the centrencephalic

circuits and ganglionic centers of the higher brain stem which play an essential role in the co-ordination of the action of the cortical areas.

*Summary.*—There is a permanent record of the stream of consciousness within the brain. It is preserved in amazing detail. No man can, by voluntary effort, call this detail back to memory. But, hidden in the interpretive areas of the temporal lobes, there is a key to a mechanism that unlocks the past and seems to scan it for the purpose of automatic interpretation of the present. It seems probable also, that this mechanism serves us as we make conscious comparison of present experience with similar past experiences.

*Conclusion.*—The discovery of this interpretive cortex is one step forward. Another territory on the map of the cerebral cortex can be named. But, seen against the vastness of the problem of the mechanisms of the mind, this is a very small step indeed.

These observations that I have reported of the temporal cortex show us another mechanism that must play its part in the total function of the human brain. It is a mechanism that can be separated out by local epileptic discharge or electrical stimulation. Like the discovery of Fritsch and Hitzig that the electrode could move the dog's leg, this discovery that the electrode can cause the past to flash into consciousness again and also provide signals of present interpretation should open a new chapter in the physiology of the brain.

This seems to constitute one of the mechanisms of consciousness. Perhaps Hippocrates would say that this is a mechanism employed by the brain to distinguish "the ugly from the beautiful, the bad from the good, the pleasant from the unpleasant."

[*] Public lecture, National Academy of Sciences, Caspary Hall, Rockefeller Institute, New York, November 18, 1957.

[1] *The First Aphorism—Hippocrates*, trans. W. H. S. Jones (London: Loeb Classical Library, 1923).

[2] C. S. Sherrington, *The Integrative Action of the Nervous System* (Cambridge: Cambridge University Press, 1947), pp. 274–276.

[3] P. Broca, "Sur la siège de la faculté du langage articulé," *Bull. soc. anat. Paris*, 2d ser. **6**, 355, 1861.

[4] G. Fritsch and E. Hitzig, "Ueber die elektrische Erregbarkeit des Grosshirns," *Arch. Anat. Physiol.*, **37**, 300, 1870.

[5] I. P. Pavlov, *Conditioned Reflexes: An Investigation of the Physiological Activity of the Cerebral Cortex*, Trans. and ed. G. Anrep (London: Oxford University Press, 1927).

[6] This case was reported by Penfield. W. Penfield, "The Cerebral Cortex in Man. I. The Cerebral Cortex and Consciousness," *Arch. Neurol. & Psychiat.*, **40**, 417, 1938; also in French (trans. Professor H. Piéron, in *l'Année psychol.* Vol. **39**, 1938).

[7] J. V. was not cured by that operation, although we managed to remove some of the abnormal area. During the twenty-one years that have followed, we have gradually learned a little more about temporal lobe epilepsy. It is now recognized as constituting the largest single group among the different forms of epilepsy. So J. V. returned for three subsequent operations, and at last she seems to be cured of the attacks.

[8] The patient is described in detail by Penfield and Jasper. W. Penfield and H. Jasper, *Epilepsy and the Functional Anatomy of the Human Brain*, [Boston: Little, Brown & Co., 1954], (Case Index, p. 882).

[9] William James, *The Principles of Psychology* (New York: Henry Holt & Co., 1910).

[10] The electric current that I have employed during recent years is produced by a square-wave generator at a frequency of 40–100 pulses per second. We usually use a pulse of 2–5 milliseconds duration and a current of 1–5 volts. It is our custom to place recording electrodes on the cortex

during stimulation, so that my associate Herbert Jasper can study the electrical condition, before and after simulation, on his electrograph placed outside the glass of the viewing stand.

[11] W. Penfield, "The Role of the Temporal Cortex in Certain Psychical Phenomena," *J. Ment. Sc.*, **101**, 451, 1955.

[12] W. Penfield, *Physiological Observations in the Human Cerebral Cortex—a Study of Conscious Responses to Stimulation of the Human Brain*, (Fifth Sherrington Lecture) (Liverpool: Liverpool University Press; Springfield, Ill.: Charles C Thomas, 1958).

[13] My associate, Sean Mullan, analyzed the cases of 217 of our patients who came to operation for relief of temporal lobe seizures (Mullan and Penfield, to be published, 1958). Of these, 38 had such interpretive illusions either at the onset of their attacks or as a result of cortical simulation or, more often, both.

# *SYMPOSIUM ON AMINO ACID ACTIVATION*

By Invitation of the Committee on Arrangements of the Autumn Meeting

*Read before the Academy, November 19, 1957*

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## *CHAIRMAN'S INTRODUCTION: SOME FACTS AND PROBLEMS**

By Fritz Lipmann

ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH, NEW YORK CITY

In a very generalizing manner, one can describe biosynthetic processes as an interplay between group activation and group transfer. In the more recent past, the process of group activation has particularly caught our interest, since it is the initial phase of all biosynthetic mechanisms. In this laboratory we have, through the years, paid much attention to such processes of group activation where a primary carrier is created, around which an often quite diverse number of secondary acceptor reactions cluster, guiding the active group into a variety of biosynthetic products.[1] Against the background of these experiences, we were primarily interested in the initial group activation, hoping that, once the problem of amino acid activation was solved, the whole complex of protein synthesis would fall into line.

From the beginning, it seemed rather likely that the primary activation in the process of protein synthesis would occur by phosphorylation of the carboxyl end of the amino acid. In an attempt to rationalize this suggestion, I proposed quite a while ago a scheme of polypeptide synthesis[2] which, in its essentials, appears now to be confirmed, and, as it has some historical interest, it is here reproduced unchanged in Figure 1.

The initial step was visualized as activation by transfer of terminal phosphate from ATP to the amino acid. This was then, and remained for some time, the only form of group activation that we knew of. Only relatively recently, initiated by some work on the finer mechanism of acetate activation,[3] did we become aware that there exists another manner of utilizing the phosphate bond energy of nucleotide triphosphate. The two now known dominant pathways are illustrated in Figure 2. The "older" process, called there *Type 2*, represents an approach of the molecule which wants to be activated to the terminal *P*, causing activation by displacement of nucleotide diphosphate by *X*. The "newer" *Type 1* was recognized, through the appearance of pyrophosphate, as a product of reaction in various activation

**Protein synthesis**

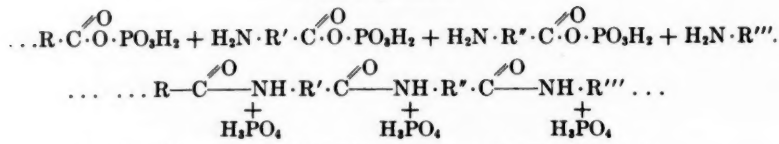


FIG. 1.—Scheme from F. Lipmann, *Advances in Enzymol.*, 1, 99, 1941.

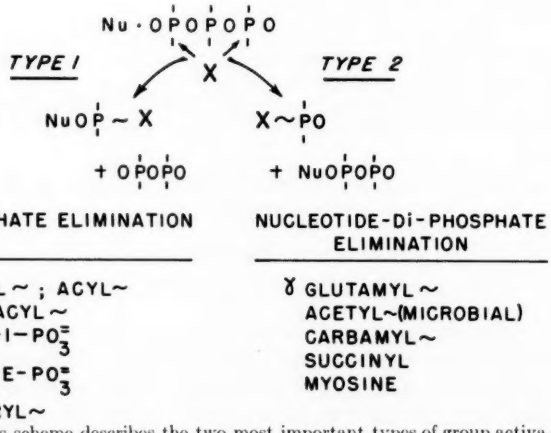


FIG. 2.—This scheme describes the two most important types of group activation through reactions with nucleotide triphosphate. In many cases, activation and transfer are synchronized on the same enzyme and the phosphorylated intermediary is not well defined. This is the case with glutamate activation for glutamine synthesis, as well as succinate activation for syccinyl-CoA synthesis, shown on the right side of the scheme, and it certainly is true also for myosin in the same column.

processes;[3, 4] it subsequently emerged that, through PP-displacement, in such cases a Nu P ~X forms, and thus the activated molecule carries along the nucleotide.[5, 6] Since no evidence had come forward for a general *Type 2* activation of amino acids, but, on the other hand, the peptic bond in pantothenic acid was shown to follow a *Type 1* displacement,[4] in a recent scheme I then proposed a *Type 1* process for amino acid activation. This more elaborate scheme for amino acid activation and peptide synthesis[7, 8] furthermore, included some suggestions toward a mechanism for a specific lining-up of amino acids.

Some merit of this rather crude model was its switch to a *Type 1* reaction as the general means of amino acid activation. This proposition was taken up by Dr. Mahlon Hoagland. He, as most of you know, while working in Paul Zamecnik's laboratory, discovered the presence of such amino acid-activating enzymes in liver extract[9, 10] and, in particular, in those fractions which would complement amino acid incorporation into microsomes. Furthermore, DeMoss *et al.*[11] showed that such amino acid activation could be found in practically all bacteria and that lencyl

adenylate, which they synthesized, reacted as predicted with this fraction in reverse direction (cf. Fig. 3).

AMINO ACID ACTIVATION

HYDROXAMATE FORMATION

$$R\cdot CHNH_2\cdot C(=O)O^- + ATP + NH_2OH \longrightarrow R\cdot CHNH_2\cdot C(=O)NHOH + PP + AMP$$

PP-EXCHANGE

$$R\cdot CHNH_2\cdot C(=O)O^- + APP\overset{x\,x}{P} \longleftrightarrow (AP\sim COCHNH_2R) + \overset{x\,x}{PP}$$

FIG. 3.—Formulation of two alternate methods used for assay of amino acid activating enzymes.

Tests for this general amino acid activation may be made in two different ways: these are formulated in Figure 3. The first assay method makes use of hydroxylamine as a trapping reagent for activated carboxyls.[12] The second test depends on the reversibility of the reaction between ATP and amino acid.[9, 11] This may be measured by the amino acid–dependent exchange between adenosine triphosphate and radioactive pyrophosphate. I have to mention, however, that the forward reaction expected from the second equation in Figure 3, namely, formation of amino acid adenylate from ATP and amino acid, is proved largely indirectly by trapping the "active" amino acid with hydroxylamine. The requirement for a rather high concentration of hydroxylamine to get this trapping effect has been interpreted as indicating that the amino acyl adenylate is in some way buried in the enzyme.[10] The finer mechanism of the reaction still appears to be somewhat obscure.

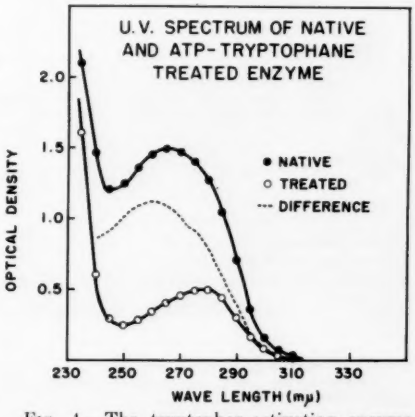


FIG. 4.—The tryptophan-activating enzyme used was approximately 50 per cent pure enzyme, as judged from ultracentrifuge versus activity data. Further details are found in Davie *et al.*, from whose work this figure is taken.

At this stage we became intensely interested in these amino acid-activating enzymes. Drs. Davie and Koningsberger in our laboratory succeeded in isolating a tryptophan-activating enzyme[13] which happens to be abundant in beef pancreas. The preparation obtained is specific for tryptophan and analogues, as shown in Table 1. A tyrosine specific activating enzyme has also been isolated from various sources.[14, 15] A specificity of such enzymes for a particular amino acid is therefore,

TABLE 1*

SUBSTRATE SPECIFICITY FOR HIGHLY PURIFIED TRYPTOPHAN-ACTIVATING ENZYME

| Substrate | Hydroxylamine (μMoles/Mg/Hr) | Exchange (μMoles/Mg/Hr) |
|---|---|---|
| L-Tryptophan | 86 | 400 |
| DL-Tryptazan | 48 | 64 |
| N-Acetyl-DL-tryptophan | 0 | ... |
| Glycyl-L-tryptophan | 0 | ... |
| D-Tryptophan | 0 | ... |
| L-Tyrosine | 3 | 8 |
| L-β-Alanine | 0 | 8 |
| L-Leucine | 0 | 0 |
| Glycine | 0 | 0 |
| L-Cysteine | 0 | 0 |

* From Davie *et al.*, *Arch. Biochem. and Biophys.*, **65**, 21, 1956.
The slight contamination with tyrosine and phenyl alanine–reactive enzyme was absent in other preparations. All preparations, however, showed reactivity with such tryptophan analogues as were incorporated into protein (cf. n. 18).

TABLE 2

AMINO ACID–DEPENDENT $PP^{32}$-ATP EXCHANGE: ACTIVATION OF ALL COMMON AMINO ACIDS BY PIGEON PANCREAS EXTRACTS

(Average of Three Experiments)

| | Δ mμM $PP^{32}$ per mg. Protein* | | Δ mμM $PP^{32}$ per mg. Protein* |
|---|---|---|---|
| Arginine | 2.3 (1–3) | Lysine | 44.0 |
| Glutamic acid | 4.0 (0–9) | Cysteine | 46.0 |
| Glutamine | 4.4 | Aspartic acid | 53.0 |
| Asparagine | 8.1 | Tyrosine | 55.0 |
| Glycine | 12.0 | Alanine | 58.0 |
| Methionine | 19.0 | Valine | 65.0 |
| Phenylalanine | 26.0 | Tryptophan | 105.0 |
| Isoleucine | 26.0 | Threonine | 108.0 |
| Serine | 32.0 | Histidine | 142.0 |
| Leucine | 35.0 | Proline | 179.0 |

Amino acid–dependent $PP^{32}$-ATP exchange was measured as described by Davie *et al.* The non-particulate supernate of 6–8-week-old pigeon pancreas was used, prepared as described by Weiss *et al.*
* Background exchange without amino acids: 18–20 mμM $PP^{32}$/mg protein.

TABLE 3*

TRANSFER OF CARBOXYL-LABELED LEUCINE TO POLYNUCLEOTIDE "FACTOR"

| Incubation Mixture | Total Counts per Minute Washed Acid-Alcohol ppt. | Phenol-Extracted Non-dialyzable |
|---|---|---|
| Heated supernatant + "factor" | 92 | 103 |
| Supernatant, 0.25 ml. alone | 49 | 43 |
| Supernatant, 0.25 ml. + 0.4 ml. "factor" (= 5 ml. super.†) | 2,060 | 1,993 |
| Same | 26 (hot TCA wash) | ... |

* From Weiss *et al.*
† Concentrate after phenol extraction. Both the preparation from pigeon pancreas, and the assay methodology are described by Weiss *et al.*

TABLE 4

INCORPORATION OF LABELED TRYPTOPHAN INTO NUCLEOTIDE ON TRYPTOPHAN-ACTIVATING ENZYME FROM BEEF PANCREAS

| Incubation Mixture | Total Counts/Min/Mg Acid-Ethanol Ppt. Direct | Phenol-extracted |
|---|---|---|
| Boiled enzyme | 825 | ... |
| Enzyme alone | 29,400 | 29,400 |
| "Factor" alone | 910 | ... |
| Enzyme + "factor" | 17,800 | 18,000 |
| Enzyme + "factor," no ATP | 1,280 | ... |
| Enzyme + "factor," no PP-ase + PP | 1,260 | ... |

A purified enzyme of the type described by Davie *et al.* was used. The two preparations used for counting are analogous to the ones used in the experiments described in Table 3 (cf. n. 17).

it seems, most likely. It also was shown in both cases that the same enzyme is responsible for amino acid hydroxamate synthesis and for the amino acid-dependent pyrophosphate exchange.

Very briefly I would like here to mention that, in most extracts from the various sources, only a certain number of amino acids were found to be activated. Our own experience with pancreas extract made us suspect that this was due to experimental difficulties, because the degree of activation of the different amino acids was variable not only with pancreas from different organisms but also from batch to batch. Lately, Drs. Aes and Weiss in this laboratory have been able to demonstrate with pigeon pancreas extract that all 20 amino acids are activated. This is shown in Table 2 and furnishes a welcome additional confirmation of the role of these enzymes in protein synthesis.

Returning now to the tryptophan-activating enzyme, we made observations in the course of its isolation which may now prove to be relevant. Although the enzyme could be highly purified, e.g., to judge by ultracentrifuge data, to about 70 per cent pure enzyme, during this purification it carried along about 4–5 per cent of an obviously tightly bound nucleotide which was not TCA-precipitable and was also largely dialyzable. It could not be removed by charcoal or Dowex 1 treatment. The only manner in which Dr. Koningsberger eventually succeeded in getting most of the nucleotide off the protein, as shown in Figure 4, was to incubate the enzyme with tryptophan and ATP. However, after such treatment, the activity of the enzyme in the hydroxamate or pyrophosphate exchange tests had not changed;[13]

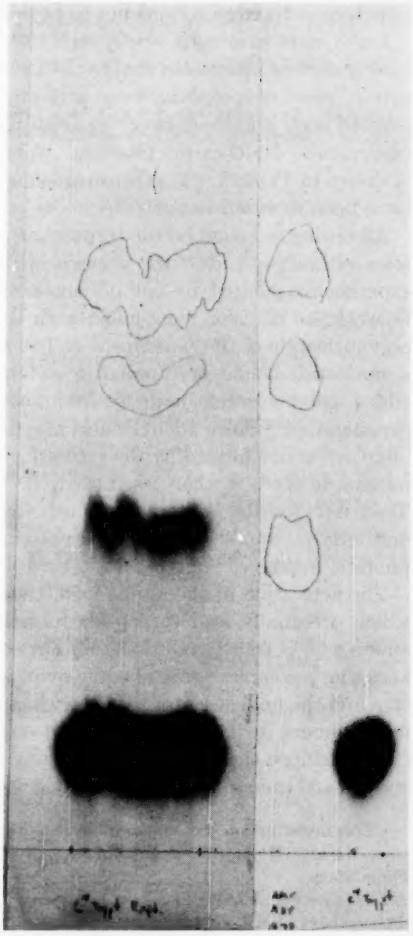

FIG. 5.—Radio autograph of pH 4.5 citrate buffer; paper electrogram made from incubate of tryptophan-activating enzyme + ATP + radioactive tryptophan. Enzyme was precipitated by acidification to pH 5, and supernate was used for paper electrogram. Radioactivity, indicating bound tryptophan, and quenching due to nucleotide, can be shown to be superimposed if the fluorescence of the tryptophan is not too strong. The tryptophan-carrying nucleotide migrates slightly above AMP; tryptophanyl-adenylate, which could be formed in the reaction, migrates in the opposite direction.

On the next to last place, markers were included for ATP, ADP, and AMP. On the last, a marker for radioactive tryptophan. The encircled areas indicate quenching as observed under Mineralight.

therefore, activation seemed not to be dependent on this nucleotide.

I now want to amplify briefly on the recent very revealing observations of Hoagland *et al.*[16] which showed that in the liver system a non-particulate polynucleotide carrier was interposed between activating enzyme and microsome. Dr. Weiss[17] isolated such a nucleotide carrier from pigeon pancreas extract and confirmed the observations on its carrier function. A transfer of the amino acid to this nucleotide is shown in Table 3. Further details on amino acid incorporation in the pancreas have been reported recently.[18]

All during our work on the tryptophan-activating enzyme, we had been searching for a natural acceptor of the amino acid. Now, obviously, the Hoagland-Zamecnik experiments pointed to this polynucleotide as an acceptor. When it was tried, however, to transfer tryptophan with the specific purified enzyme to the carrier polynucleotide of the experiment of Table 3, it appeared, as shown in Table 4, that a nucleotide-bound tryptophan was formed with activating enzyme alone, and added polynucleotide actually inhibited. This unexpected result is now under investigation. Since adenylic acid also appears to be incorporated into the tryptophan derivative formed by the activating enzyme, its relation to the enzyme-bound nucleotide needs further substantiation. It may, at present, be described as most likely representing an initial product of the interaction between ATP, tryptophan, and enzyme, more stable than tryptophanyl adenylate, and of opposite electrophoretic mobility.

The activation of the amino acid, then, appears to initiate a chain of reactions which eventually lead to peptide formation in the microsome. On its way, the amino acid is transferred first to a nucleotide and one may state with some assurance that a complex array of carry-overs is interposed between the initial activation step and the final acceptor reaction when the amino acid actually is connected with its neighbors in the protein. These obviously represent parts of the machinery that produces the sequential arranging of the amino acids and determines the specific and unique features of a particular protein.

* This investigation was supported in part by a research grant from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service; and the National Science Foundation.

The following abbreviations have been used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; PP, pyrophosphate; P, phosphate; NuP, nucleotide monophosphate; A, adenosine.

[1] F. Lipmann, *Science*, **120**, 855, 1954.

[2] F. Lipmann, *Advances in Enzymol.*, **1**, 99, 1941.

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[7] F. Lipmann, *Mechanism of Enzyme Action*, ed. W. D. McElroy, and B. Glass (Baltimore: Johns Hopkins Press, 1954), p. 599.

[8] F. Lipmann, *Currents in Biochemical Research*, ed. D. E. Green, (1956), p. 241.

[9] M. B. Hoagland, *Biochim. et Biophys. Acta*, **16**, 228, 1955.

[10] M. B. Hoagland, E. B. Keller, and P. C. Zamecnik, *J. Biol. Chem.*, **218**, 345, 1956.

[11] J. A. DeMoss, S. M. Genuth, and G. D. Novelli, these PROCEEDINGS, **42**, 325, 1956.

[12] F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **161**, 415, 1945.

[13] E. W. Davie, V. V. Koningsberger, and F. Lipmann, *Arch. Biochem. and Biophys.*, **65**, 21, 1956.

[14] V. V. Koningsberger, A. M. van de Ven, and J. T. G. Overbeck, *Royal Dutch Acad. Sci.*, Ser. B **60**, 141, 1957.

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[16] M. B. Hoagland, P. C. Zamecnik, and M. L. Stephenson, *Biochim. et Biophys. Acta*, **24**, 215, 1957.

[17] S. B. Weiss, G. Acs, and F. Lipmann, these PROCEEDINGS, **44**, 189, 1958.

[18] N. Sharon and F. Lipmann, *Arch. Biochem. and Biophys.*, **69**, 219, 1957.

## *INTERMEDIATE REACTIONS IN AMINO ACID INCORPORATION**

BY P. C. ZAMECNIK, M. L. STEPHENSON, AND L. I. HECHT

JOHN COLLINS WARREN LABORATORIES OF THE HUNTINGTON MEMORIAL HOSPITAL OF HARVARD UNIVERSITY, AT THE MASSACHUSETTS GENERAL HOSPITAL

As a result of work carried on in several laboratories, including our own, it has appeared until recently that the process of incorporation of labeled amino acids into protein could be divided into three parts.[1] At the present time it seems likely that another subdivision may be made, as illustrated in Figure 1. Evidence has

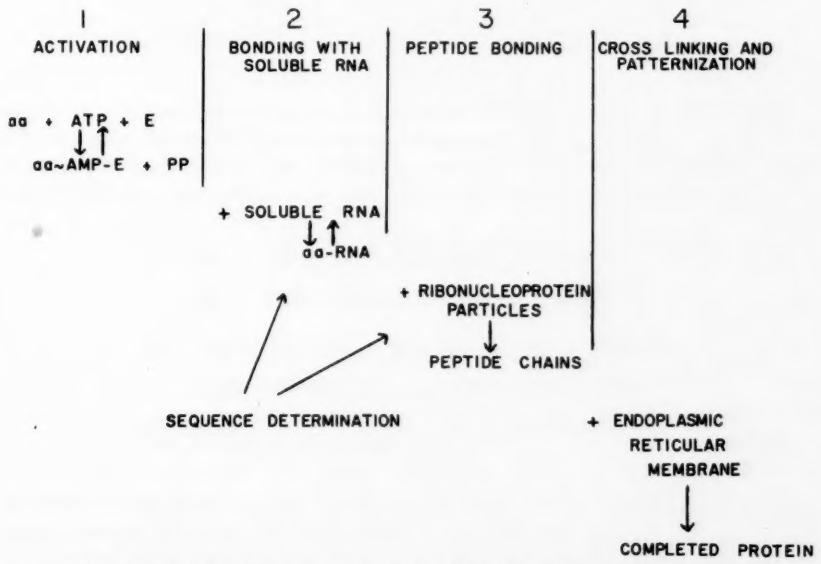


FIG. 1.—Postulated Steps in incorporation of labeled amino acids into proteins.

accumulated that a soluble RNA fraction of cell cytoplasm serves as an intermediate in the incorporation of labeled amino acid into protein.

Our interest in this soluble RNA fraction was aroused in a devious way. Some

time ago we wished to inquire whether the same cell-free system in which amino acids were incorporated into protein would serve to incorporate nucleotide precursors into RNA. We found that 8-$C^{14}$-ATP (but not 8-$C^{14}$-ADP) served very well as a precursor for the formation of labeled RNA in this system. As a control, we added $C^{14}$-leucine rather than $C^{14}$-ATP. Isolation of the RNA in this case revealed that the $C^{14}$-leucine had also become bound to the RNA. Fractionation of the cellular RNA in such a system indicated that the maximal labeling of RNA by the $C^{14}$-leucine occurred in the pH 5 precipitable portion of the 105,000 $\times$ *g.* supernate.[2] This same cellular fraction is active in the incorporation of mononucleotides into the terminal residues of the RNA located therein, when $C^{14}$-ATP, $C^{14}$-UTP, and $C^{14}$-CTP are employed as precursors,[3–5] as shown in Table 1. The

TABLE 1

MONONUCLEOTIDE TRIPHOSPHATES AS PRECURSORS OF INCORPORATION OF MONONUCLEOTIDE RESIDUES INTO RNA*

| | mμMoles per Mg. RNA |
|---|---|
| ATP-$C^{14}$ | 14.3 |
| ATP-$C^{14}$ + CTP | 36.8 |
| ATP-$C^{14}$ + CTP + GTP + UTP | 39.4 |
| CTP-$C^{14}$ | 37.3 |
| CTP-$C^{14}$ + ATP + GTP + UTP | 38.0 |
| UTP-$C^{14}$ | 3.0 |

* The nucleotides (1 μmole/ml) were incubated for 10 minutes at 37° C. with 0.5 ml. of pH 5 fraction prepared from ascites cells. The medium contained 0.01 *M* phosphopyruvate; 0.004 *M* $MgCl_2$; 0.037 *M* Tris buffer, pH 7.6; 0.020 *M* KCl; and 10 μg. pyruvate kinase/ml. The specific activity of the labeled nucleotides was 243,000 c.p.m./μmole ATP; 58,000 c.p.m./μmole CTP; and 200,000 c.p.m./μmole UTP.

mechanism of the mononucleotide incorporation reactions, as illustrated in Figure 2, involves a common pattern of pyrophosphate cleavage, thus differing from the polynucleotide phosphorylase reaction of Ochoa and co-workers.[6] Since these reactions of Figure 2 appear to be reversible, they suggest that the soluble RNA may

$$C^{*}RPPP + RNA \rightleftharpoons C^{*}RP\text{-}RNA + PP$$

$$U^{*}RPPP + RNA \rightleftharpoons U^{*}RP\text{-}RNA + PP$$

$$A^{*}RPPP + CRP\text{-}RNA \rightleftharpoons A^{*}RP\text{-}CRP\text{-}RNA + PP$$

$$P^{*}P^{*} + CRP\text{-}RNA \rightleftharpoons CRPP^{*}P^{*} + RNA$$

FIG. 2.—Mechanism of incorporation of mononucleotides into soluble RNA

serve as a storehouse for releasable nucleotide coenzymes, which play a directive role in regulation of the metabolism in a particular type of cell and logically also in its progeny, equipped with the same type of RNA. Whether there is a link between the nucleotide and amino acid incorporations into the soluble RNA of the pH 5 precipitable fraction remains a question for the future.

As to the nature of this soluble RNA, it has an apparent molecular weight of the order of 10,000, is polydisperse in the ultracentrifuge,[7] and needs more precise characterization.

Returning to the role of this soluble RNA in incorporation of amino acids into protein, our present impression is that the labeled amino acid–RNA compound is an intermediate on the path to completed protein, as illustrated in Figure 1. If such is the case, it becomes logical to inquire whether enzymes participate in this additional step, as illustrated in Figure 3. On this basis, one might postulate the presence of at least four separate enzymes in the over-all incorporation reaction. The first two steps in the incorporation reaction can be studied in the absence of microsomes. The first step involves activation of amino acids by way of the aminoacyl-adenylate intermediate postulated by Hoagland.[8, 9]

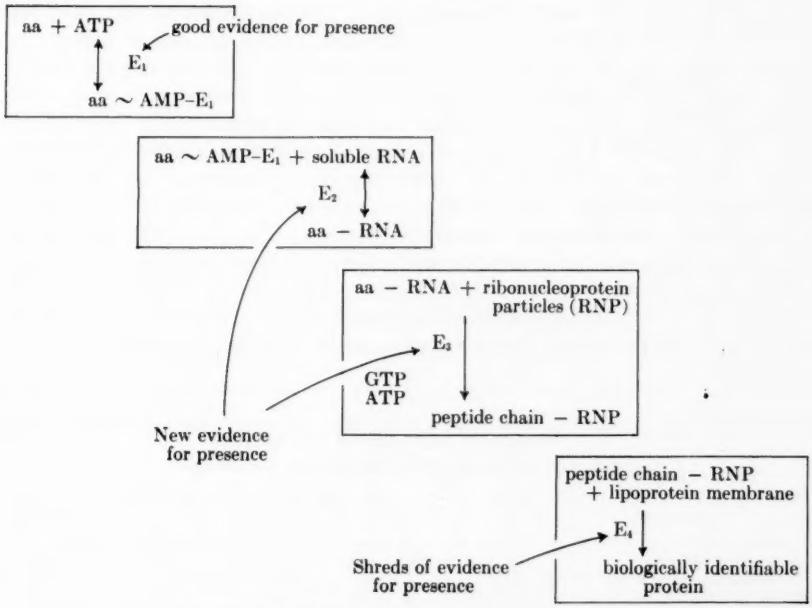


FIG. 3.—Possible enzymes involved in protein synthesis

Support for this aminoacyl-adenylate concept has come from the transfer of $O^{18}$ from tryptophan, labeled with $O^{18}$ in the carboxyl group, to AMP, in the presence of ATP, tryptophan-activating enzyme, and hydroxylamine.[10] This indicates a direct interaction of the tryptophan with the phosphate of AMP, in consonance with a mixed anhydride intermediate. Evidence for the presence of such a free aminoacyl-adenylate has been lacking, and it has therefore been considered to be tightly enzyme-bound. In recent experiments from this laboratory, it has, however, been found that if $C^{14}$-valine is incubated with ATP and the pH 5 fraction in the presence of a large excess of inert valyl-AMP as a trapping agent, labeled valyl-AMP may be isolated in small amount.[11]

These two pieces of evidence, plus the lack of sensitivity of the amino acid–activating reaction to ribonuclease, point toward a first step in the over-all incorporation reaction which does not involve RNA. This soluble RNA is, however,

closely associated with the pH 5–activating enzymes and may be part of a reaction sequence bound together in the intact cell.

For the present we may consider the interaction with soluble RNA to be a second step in the sequence. If the leucine-labeled RNA is to be considered as a true intermediate in the over-all scheme rather than as a storage locus for activated amino acids, it should satisfy certain criteria of intermediates. It should, for example, be more rapidly labeled than are the peptide chains in the ribonucleoprotein particles. Such was found to be the case in short time experiments carried out on intact ascites tumor cells.[7] The labeling of soluble RNA by the amino acid was such a rapid reaction that it was difficult to obtain a satisfactory time curve, even when the reduced temperature of 25° C. was employed. Another criterion for an intermediate is that it should when labeled, be, able to transfer its labeled amino acid to the ribonucleoprotein particles. Leucine-labeled soluble RNA was therefore prepared and isolated by a phenol extraction procedure. Addition of this labeled soluble RNA to microsomes in the presence of GTP and ATP and a small aliquot of a dilute supernatant fraction resulted in transfer of the labeled leucine to peptide bonding in the ribonucleoprotein particles.[7] The additional protein from the supernatant cell fraction required to mediate this transfer is designated $E_3$. In the crude separations presently employed, this enzyme is located both in the pH 5-precipitable and in the pH 5-supernatant (or $S_4$) fraction. GTP participates as a cofactor in this reaction sequence and cannot be replaced by UTP or CTP.[2] Another piece of evidence pointing to soluble RNA as an intermediate in the over-all incorporation reaction is shown in Table 2. In the

TABLE 2

INCORPORATION OF $C^{14}$-LEUCINE INTO RNA AND PROTEIN OF ASCITES CELL FRACTIONS AFTER INCUBATION OF FRACTIONS WITH $C^{14}$-LEUCINE*

Effect of Added pH 5 RNA on the Incorporation

| | TOTAL COUNTS IN FRACTIONS | | | |
|---|---|---|---|---|
| | RNA-Amino Acid | | Protein | |
| Added RNA | 0 | 120 μg. | 0 | 120 μg. |
| *Complete system:* | | | | |
| $S_4$ + microsomes | 136 | 927 | 136 | 357 |
| $S_4$ + microsomes + pH 5 fraction | ... | 918 | ... | 321 |
| *Partial systems:* | | | | |
| $S_4$ | ... | 0 | ... | 5 |
| Microsomes | 93 | 240 | 62 | 82 |
| pH 5 fraction | 0 | 210 | 4 | 12 |
| pH 5 fraction + microsomes | 255 | 584 | 137 | 224 |

* A 10 per cent lysate of ascites cells was prepared according to the method of Littlefield and Keller[12]. The fractions were separated by differential centrifugation. The fraction sedimenting after 10 minutes at 12,000 × *g*. was discarded, and the supernatant was centrifuged for 2 hours at 105,000 × *g*. in a Spinco preparative ultracentrifuge. The microsome pellet was resuspended in one-tenth the original volume of medium. The 105,000 × *g*. supernatant was brought to pH 5.2 with *N* acetic acid, and the resultant precipitate was isolated by centrifugation. This pH 5 fraction was resuspended to the original volume in the medium. The 105,000 × *g*. supernatant minus the pH 5 fraction (supernatant 4, or $S_4$) was readjusted to pH 7.4. The amount of each fraction incubated was as follows: microsomes 1.72 mg. dry protein, 438 μg. RNA; pH 5 fraction, 0.81 mg. protein, 30 μg. RNA; $S_4$, 1.08 mg. protein, and 5.2 μg. RNA. After incubation both RNA and protein were isolated and counted (see n. 7).

Each flask contained 1 μmole ATP; 0.1 μmole $C^{14}$-leucine containing 139,000 c.p.m.; 10 μmoles phosphopyruvate; 0.01 mg. pyruvate kinase; 0.25 μmole GTP plus the cell fractions containing 3.0 μmoles $MgCl_2$; 10 μmoles KCl; 35 μmoles Tris, pH 7.6; and 300 μmoles sucrose. The total volume was 1.5 ml. Where indicated, 120 μg. liver pH 5 RNA was added. The RNA was prepared from liver pH 5 fraction by a modification of the phenol methods (see nn. 7, 13, 14). Incubation was for 10 minutes at 37° C.

complete system (first line, Table 2), the addition of previously isolated soluble RNA produces a marked stimulation of the incorporation of leucine into protein, and likewise a striking increase in the total RNA-bound $C^{14}$-leucine. A similar type of stimulation of incorporation of amino acid into protein had already been

observed in unpublished experiments by Weiss and Lipmann.[15] In our case, it became possible to demonstrate this stimulating effect of RNA on amino acid incorporation into protein by employing the so-called $S_4$ fraction, which is relatively free from endogenous RNA. The $S_4$ fraction alone is low in leucine activating enzyme ($E_1$) and therefore is itself unable to catalyze the two-step leucine labeling of RNA. It is rich in $E_2$ and $E_3$, as evidenced by its stimulation of RNA-labeling and incorporation of labeled amino acids into protein in the presence of microsomes. The microsomes alone contain (or have not been freed from) sufficient amounts of activating enzymes ($E_1$) to catalyze maximal RNA and protein-labeling without addition of pH 5 fraction, provided that $S_4$ is present. The relationship of the postulated enzymes in the three cell fractions which concern us here is illustrated in Figure 4.

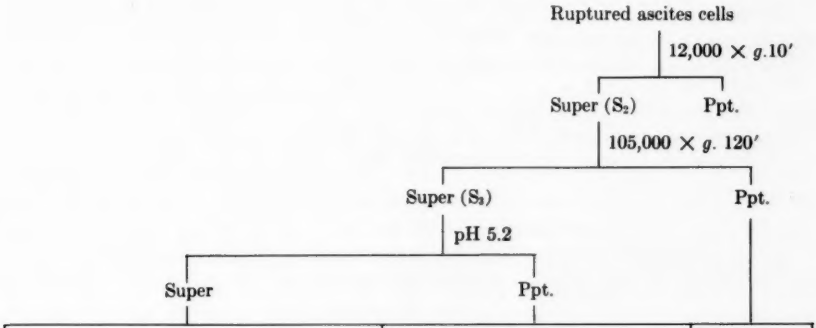


FIG. 4.—Location of enzymatic components of leucine incorporation system in ascites tumor fractions

It is also possible that determination of the amino acid sequence may be a two-step RNA-participating process.

*Summary.*—In summary, it may be said that further fractionation of cell-free systems from the mouse Ehrlich ascites tumor and the rat liver points toward the participation of soluble RNA in the incorporation of amino acid into protein and indicates that there may be at least three separate enzymatic steps from free amino acid to peptide bonding in the ribonucleoprotein particles.

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The following abbreviations are used: RNA, ribonucleic acid; ATP or ARPPP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; UTP or URPPP,

uridine triphosphate; CTP or CRPPP, cytidine triphosphate; and GTP, guanosine triphosphate.

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## *AN ENZYMATIC MECHANISM FOR LINKING AMINO ACIDS TO RNA**

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### INTRODUCTION

For the past several years we have been interested in the general problem of acyl group activation. Our initial studies were concerned primarily with the enzymatic mechanism of acetyl CoA formation.[1, 2] The results of these investigations demonstrated the existence of a new type of reaction in which the carboxyl group of acetate reacts with ATP and becomes linked to the phosphate of adenosine-5′-phosphate. This "activated" acetyl group is subsequently transferred, by the same enzyme, to coenzyme A (eqs. [1]–[3]).

$$\text{ATP} + \text{acetate} \rightleftharpoons \text{adenyl acetate} + \text{PP} \quad (1)$$

$$\text{Adenyl acetate} + \text{CoA} \rightleftharpoons \text{acetyl Coa} + \text{AMP} \quad (2)$$

$$\text{ATP} + \text{acetate} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{AMP} + \text{PP} \quad (3)$$

The widespread occurrence of this type of reaction in the activation of higher fatty acids,[3–6] inorganic acids,[7–9] and other acyl compounds[10, 11] has been demonstrated by a number of investigators. Of particular interest was the finding by Hoagland et *al.*[12] and others[13–16] of enzymes which carry out a reaction analogous to that shown in equation (1) with amino acids. Purification and characterization of some of these enzymes showed, first, that the enzymes were specific for a single amino acid and, second, that the product of the reaction was the adenyl amino acid

presumably existing tightly bound to the enzyme.[13–16] Direct evidence supporting the hypothesis of adenyl amino acid formation was provided by the chemical synthesis of several adenyl amino acids and the demonstration of their conversion to ATP in the presence of PP and the appropriate enzyme.[17–19]

One of the major questions concerning the metabolism of adenyl amino acids is their biologic function. One hypothesis suggests that these amino acid derivatives are the immediate precursors of protein synthesis, and support for this idea is derived from studies of amino acid incorporation into protein by subcellular particles.[12] Our approach to the question of adenyl amino acid utilization was directed toward identifying the immediate acceptor of the "activated" amino acid. The information obtained from our earlier studies on acetate activation suggested that the enzymes which carry out the formation of the adenyl amino acids might also catalyze the transfer of the amino acid to a suitable acceptor. We felt that the isolation and characterization of such an acceptor would provide information pertinent not only to the details of adenyl amino acid formation but perhaps also to the mechanism of protein synthesis.

In the present paper we shall describe an enzymatic reaction which links amino acids to ribonucleic acid (RNA). This reaction requires, in addition to the amino acid and a specific RNA fraction, ATP, $Mg^{++}$, and the corresponding amino acid–activating enzyme. For a given amino acid, the activation reaction and subsequent transfer to RNA appear to be catalyzed by a single enzyme, i.e., the amino acid–activating enzyme. Moreover, our studies indicate that there is a fixed number of sites available for each amino acid per unit of RNA. Our hypothesis to depict this reaction is summarized by the following reactions:

$$\text{ATP} + \text{amino acid} \rightleftharpoons \text{adenyl amino acid} + \text{PP} \quad \text{(activation step)} \qquad (4)$$

$$\text{Adenyl amino acid} + \text{RNA} \rightleftharpoons \text{RNA-amino acid} + \text{AMP} \quad \text{(transfer step)} \qquad (5)$$

$$\text{ATP} + \text{amino acid} + \text{RNA} \rightleftharpoons \text{RNA-amino acid} + \text{AMP} + \text{PP} \quad \text{(over-all reaction)} \qquad (6)$$

## EXPERIMENTAL RESULTS

If one incubates a mixture of $C^{14}$-labeled amino acids with a dialyzed sonic extract of *Escherichia coli* in the presence of ATP, the amino acids are converted to an acid-insoluble form which is stable to repeated washings with cold 0.3 *M* perchloric acid. Purification of the bacterial extract revealed that two fractions are required for this conversion. One of these fractions can activate a number of amino acids, and the other, which is composed largely of ribonucleic acid, appears to function as the amino acid acceptor.

Using a single labeled amino acid as substrate, e.g., 1-$C^{14}$-valine, the system can be reconstructed with a purified valine-activating enzyme[20] and a specific RNA fraction isolated from *E. coli*. The complete requirements for the reaction are shown in Table 1. It is clear that there is an absolute requirement for ATP, RNA, and the enzyme. The effect of $Mg^{++}$ is best shown in the second experiment, where less enzyme was used and the rate rather than the final yield of the reaction was determined. Under these conditions $Mg^{++}$ increases the rate of the reaction about ten times.

*Properties of the RNA–Amino Acid Complex.*—The RNA–amino acid complex can

be precipitated with cold 0.3–0.5 *M* perchloric acid and is readily distinguished from protein-bound amino acids by the following criteria. Treatment of the acid-precipitated product with 5 per cent perchloric acid at 100° for 15 minutes or exposure to 0.01 *N* alkali at 25° for 5 minutes completely converts the amino acids to an acid-soluble form. Even much milder treatments, such as incubation in Tris buffer, pH 8.6, at 37° or exposure to hydroxylamine (pH 7.0), result in the disappearance of the amino acids from the acid-insoluble fraction (Table 2). In the presence of 0.8 *M*

TABLE 1

REQUIREMENTS FOR INCORPORATION OF $C^{14}$-VALINE*

| Exp. No. | Components | $C^{14}$-Valine Incorporated (mμmole) |
|---|---|---|
| 1 | Complete | 0.33 |
| | Minus RNA | 0.00 |
| | Minus ATP | 0.00 |
| | Minus enzyme | 0.00 |
| 2 | Complete | 0.11 |
| | Minus $Mg^{++}$ | 0.01 |

* The reaction mixture contained in a total volume of 0.25 ml., 20 μmoles of sodium cacodylate buffer pH 7.0; 1 μmole of $MgCl_2$; 0.2 μmole of ATP 0.42 μmole of $C^{14}$-DL-valine (specific activity 6.05 × $10^6$ c.p.m. per μmole) 0.15 ml. of RNA containing 12 μmoles of pentose nucleotide per ml., and 0.14 μg. of the valine-activating enzyme, having an activity of 66 units per μg. of protein in the ATP-PP exchange reaction (see Berg, *J. Biol. Chem.*, **222**, 1025, 1956). Time 10 minutes; temperature 30°. In experiment 2, 0.10 ml. of RNA and 0.03 μg. of the valine-activating enzyme were used.

TABLE 2

PROPERTIES OF RNA–AMINO ACID COMPOUND*

| Treatment | Time (Min.) | C.p.m. Linked to RNA |
|---|---|---|
| Tris buffer, pH 8.6, 37° | 0 | 2,681 |
| | 5 | 1,344 |
| | 10 | 684 |
| | 20 | 237 |
| No hydroxylamine, pH 7, 30° | 5 | 2,616 |
| 0.4 *M* | 5 | 1,489 |
| 0.8 *M* | 5 | 703 |
| 1.6 *M* | 5 | 248 |
| Product at zero time | | 2,755 |
| Cacodylate buffer, pH 7, 30° | 10 | 2,090 |
| Plus 0.10 μg/ml RNAase | 10 | 15 |
| Plus 3.0 μg/ml DNAase | 10 | 2,348 |

* *Procedure:* $C^{14}$-labeled product was isolated by precipitation with acid, and aliquots were treated as described above. The radioactivity of the resultant acid-precipitable material was determined.

hydroxylamine at pH 7 and 30°, the half-life for removal of the linked amino acids is 3 minutes, while under the same conditions in the absence of hydroxylamine the half-life is 62 minutes. Exposure of the RNA–amino acid compound to pancreatic ribonuclease completely converts the amino acids to an acid-soluble form. Similar treatment with pancreatic deoxyribonuclease has no effect, even though added DNA is almost completely degraded. Studies are now in progress to determine whether treatment with ribonuclease results in cleavage of the RNA–amino acid linkage or whether the complex is degraded to smaller acid-soluble fragments with the amino acids still attached.

The amount of amino acid incorporated into the acid-insoluble fraction is a

linear function of the amount of RNA added (Fig. 1). This is true whether $C^{14}$-labeled valine and the purified valine-activating enzyme are used (curve *A*) or whether a mixture of $C^{14}$-labeled amino acids[21] and an extract of *E. coli* are used (curve *B*). The reaction proceeds at a rate which is proportional to the amount of amino acid–activating enzyme used and then stops. Addition of more enzyme, amino acid, or ATP produces no further increase in the amount of amino acid incorporated. Addition of more RNA at this point, however, results in a further incorporation of amino acid which is proportional to the amount of RNA added. The incorporation of a given amino acid is independent of the incorporation of other amino acids. For example, with three labeled amino acids together (leucine, valine, and methionine), the total amount of radioactivity linked to the RNA is equal to the sum of the amount incorporated with each amino acid alone.

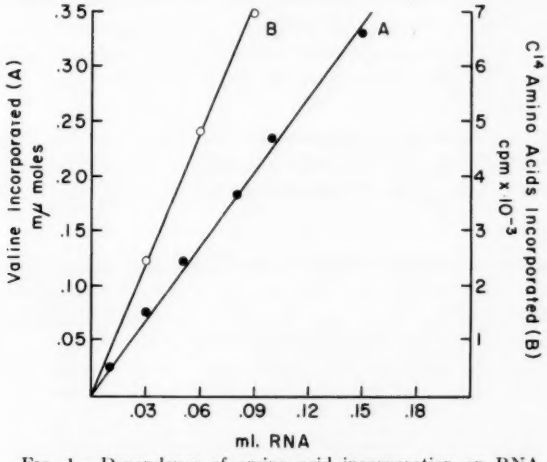


FIG. 1.—Dependence of amino acid incorporation on RNA. *Curve A:* reaction mixture same as in Table 1. *Curve B:* The reaction mixture contained in a total volume of 0.50 ml., 50 μmoles of sodium cacodylate buffer, pH 7.0; 1.5 μmoles of $MgCl_2$; 0.2 μmole of ATP; $9.0 \times 10^5$ e.p.m. of the labeled amino acid mixture,* and 0.04 ml. of dialyzed sonic extract of *E. coli* (2 mg. protein/ml). The RNA preparation used was the same as that described in Table 1. Time 20 minutes; temperature 30°. *Hydrolysate of protein from *Chromatium* grown in $C^{14}O_2$ (see n. 21). Specific activity $7.3 \times 10^5$ e.p.m. per μmole of carbon.

*Properties of the RNA.*—The preparation of RNA used in these studies was obtained from dried cells of *E. coli* by extraction with hot detergent,[22] followed by salt and alcohol fractionation, and adsorption and elution from charcoal. The ability to act as an acceptor of amino acids is not destroyed by heating at 60° for periods up to 30 minutes, nor is it significantly inpaired after precipitation at pH 2. At pH 7.0, the most purified material showed an ultraviolet absorption maximum at 260 mμ and a minimum at 230 mμ. The 260 mμ: 230 mμ ratio is 2.33, and the 260 mμ: 280 mμ ratio is 1.91. The preparation contains, relative to the pentose nucletide content, 3 per cent of deoxypentose nucleotide[23] and between 3 and 5 per cent of protein.[24]

*Specificity of RNA Acceptor.*—Using the assay system described in Figure 1, *B*, a number of RNA preparations isolated from a variety of sources have been tested for their ability to function as amino acid acceptors. These included RNA from yeast (prepared in the same way as the active RNA from *E. coli*), *Azotobacter vinelandii*, turnip yellow mosaic virus, tobacco mosaic virus, and rat liver.[25] The activity with these preparations was 5 per cent or less of that found with the RNA fraction from *E. coli*. We have also tested several enzymatically synthesized polyribonucleotides[26] such as polyadenylic acid, polyuridylic acid, polyadenylic-uridylic acid, and the mixed polymer containing all four naturally occurring nucleotides. These, too, were inert as amino acid acceptors. Moreover, not all the RNA which can be extracted from *E. coli* is active; over 50 per cent of the cellular RNA can be precipitated from the original detergent extract with sodium chloride (1.5 *M*), but this fraction has little or no activity as an acceptor of amino acids. It is not yet clear, however, whether the lack of activity with the various RNA preparations tested is due to a fundamental inability to accept amino acids or whether it is due to some difference in the physical state of the RNA. Further work is in progress to determine this point.

*Specificity of Amino Acid Incorporation.*—We have examined the question of whether an amino acid–activating enzyme is specific with regard to the amino acid which it links to RNA. Table 3, experiment 1, shows that the purified valine-ac-

TABLE 3

INCORPORATION OF AMINO ACIDS BY VALINE-ACTIVATING ENZYME AND BY EXTRACTS OF *E. coli**

| Exp. No. | Amino Acid | Amino Acid Incorporation (MμMoles per Ml. RNA) |
|---|---|---|
| 1 | Valine | 1.78 |
| | Leucine | 0.00 |
| | Phenylalanine | 0.00 |
| | Methionine | 0.00 |
| 2 | Valine | 5.44 |
| | Leucine | 14.7 |
| | Phenylalanine | 4.90 |
| | Methionine | 3.39 |
| | Tryptophane | 2.00 |

* The reaction mixture contained in a total volume of 0.25 ml., 20 μmoles of sodium cacodylate buffer, pH 7.0 1.5 μmoles of $MgCl_2$ 0.2 μmole of ATP; 0.2 μmole of $C^{14}$-DL-valine (specific activity $6.05 \times 10^6$ c.p.m per μmole); 0.2 μmole of $C^{14}$-DL-phenylalanine (specific activity $1.1 \times 10^6$ c.p.m. per μmole); 0.25 μmole of $C^{14}$-DL-leucine (specific activity $5.1 \times 10^6$ c.p.m. per μmole); 0.18 μmole of $C^{14}$-L-methionine (specific activity $2.95 \times 10^6$ c.p.m. per μmole); or 0.52 μmole of $C^{14}$-DL-tryptophane (specific activity $4.67 \times 10^6$ c.p.m. per μmole). Experiment 1 contained 0.03 μg. of the valine-activating enzyme and 0.10 ml. of the RNA preparation described in Table 1. Experiment 2 contained 0.05 ml. of the dialyzed sonic extract of *E. coli* (2 mg protein/ml) and 0.05 ml. of a more purified RNA preparation containing 12.5 μmoles of pentose nucleotide/ml. Time: 20 minutes; temperature, 30°.

tivating enzyme catalyzed only the incorporation of valine. Likewise, a purified methionine-activating enzyme,[14] which incorporated 0.87 mμmole of methionine/ml of the RNA preparation, incorporated less than 0.04 mμmole of leucine, valine, or phenylalanine. The same RNA preparation, however, can accept a number of different amino acids, provided that a source of the appropriate activating enzymes is present. With an extract of *E. coli* which can activate a large number of amino acids,[13] leucine, phenylalanine, tryptophane, methionine, and valine become linked to the RNA (Table 3, exp. 2). The extent of incorporation of each of these amino

acids is, as mentioned earlier, dependent on the amount of RNA present. Thus it appears that the incorporation of a given amino acid onto RNA requires the corresponding amino acid–activating enzyme.

Of particular significance in understanding this reaction is the question of whether the amino acid–activating and –incorporating activity are due to the same enzyme. To study this question, we examined the methionine-activating enzyme[14] at various stages of purity for activity with respect to methionine activation and incorporation onto RNA (Table 4). The activation step may be determined separately by meas-

TABLE 4

COMPARISON OF METHIONINE ACTIVATION AND INCORPORATION ACTIVITY AT VARIOUS STAGES OF PURIFICATION*

| Fraction | Specific activity (Units per Mg. of Protein) | Activity A† (Units per Ml.) | Activity B‡ (Units per Ml.) | A/B |
|---|---|---|---|---|
| Crude extract | 0.20 | 4.5 | 6.1 | 0.74 |
| A-2 | 5.4 | 23.1 | 33.2 | 0.70 |
| AS-1 | 9.2 | 15.7 | 23.2 | 0.68 |
| AS-2b | 15 | 17.5 | 24.2 | 0.72 |
| C$\gamma$ eluate | 19 | 2.7 | 3.9 | 0.69 |

* A detailed description of the purification procedure will be published at a later date. The specific activity is expressed as units of methionine activation activity (Activity B) per mg. of protein (see n.14).

† Activity A refers to the incorporation of methionine onto RNA expressed as units per ml. One unit of activity is equal to the incorporation of 0.34 m$\mu$mole of methionine in 10 minutes at 30°.

‡ Activity B refers to the methionine-activation activity expressed as units per ml. One unit of activity is equal to the incorporation of 1 $\mu$mole of $PP^{32}$ into ATP in 15 minutes in the standard exchange assay described previously (see n. 14).

uring the methionine-dependent exchange of $PP^{32}$ with $ATP^{14}$, and the incorporation reaction was measured as described earlier. It is clear that, over an approximately hundred fold purification of the enzyme, the two activities were purified to the same extent. We have concluded from these data that the amino acid-activating enzyme also catalyzes the transfer of the activated amino acid to the RNA.

## DISCUSSION

We have provided evidence in this communication for an enzymatic reaction linking amino acids to RNA. The resemblance of this mechanism to that demonstrated for acyl CoA formation is of some interest. In each case the formation of the adenyl acyl derivative and the subsequent transfer of the acyl moiety to an appropriate acceptor appear to be carried out by a single enzyme. Such a mechanism would tend to stabilize the relatively labile adenyl amino acid intermediate by combination with the enzyme until, in the presence of RNA, it is converted to the more stable RNA-amino acid derivative. We have also carried out some experiments to determine whether, like the acetate activation reaction, amino acid incorporation onto RNA is reversible. Although these experiments are not yet complete, it is clear that the bound amino acids are readily removed from the RNA in the presence of AMP and PP, resulting in the formation of ATP (see eqs. [4]–[6]). This observation may explain the finding by Holley[27] of an amino acid–dependent, RNAase-sensitive, exchange of AMP-$C^{14}$ with the adenyl moiety of ATP. Experiments are now in progress with the synthetic adenyl amino acids to obtain more detailed information on the mechanism and specificity of the amino acid transfer to RNA.

The significance of the present observations on the linking of amino acids to RNA with regard to the mechanism of protein synthesis is not clear. Many of the current ideas concerning protein synthesis visualize this process as involving, first, the

activation of the free amino acids to some higher-energy level, followed by the orientation of these activated amino acids in a predetermined sequence on a nucleic acid "template." Such sequentially arranged amino acids then undergo polymerization and eventual conversion to a polypeptide chain having a specific configuration.[28–32] The most attractive hypothesis at present, although by no means the only one, is that the linking of amino acids to RNA represents the so-called template stage mentioned above. Consistent with this view is the finding by Hoagland *et al.*[33] that the amino acids of an RNA–amino acid compound from rat liver are incorporated into peptide linkage by the liver microsome fraction. Although the evidence for this view is still incomplete, it is of some interest to consider our present information with respect to the template hypothesis.

Some of the questions which are posed by the template hypothesis are: (1) What is the nature of the linkage between the amino acid and the nucleic acid? (2) What is the minimal size of the structural unit required to bind a single amino acid? (3) What are the determinants which specify the positioning of a given amino acid on the nucleic acid?

With regard to the first question, we do not yet have sufficient data to describe the precise chemical linkage between the amino acids and the RNA. The marked lability of the isolated RNA–amino acid complex to alkali and hydroxylamine and the relative stability at low pH closely resemble that found with the synthetic adenyl amino acids.[17, 18] The major difference appears to be the slightly greater stability of the RNA–amino acid compound at neutral pH. This resemblance suggests that the amino acid carboxyl groups are linked to the phosphates of the RNA If such is the case, the amino group of an adjacent linked amino acid might readily attack the acyl phosphate linkage to form a peptide bond. Wieland *et al.*[19] have reported that mixing of an adenyl amino acid with free amino acids leads to the formation of peptides in which the carboxyl group of the activated amino acid is transferred to the amino group of the second amino acid.

Our experiments suggest that there are a fixed number of sites specific for a given amino acid associated with the RNA. Under the conditions we have used, these sites appear to function independently, that is, there is no enhancement or inhibition of incorporation when more than one amino acid is used as substrate. Our studies thus far do not enable us to make any estimate of the minimal size of the amino acid–binding site. From the extent of incorporation observed with 5 amino acids (Table 3, exp. 2), it appears that one amino acid is bound per approximately 400 nucleotide units. This figure of approximately 400 nucleotides is, of course, maximal, and further studies with more amino acids and more purified RNA preparations are required to arrive at the minimal figure.

The question of what structural features of the RNA determine the positioning of the amino acids has been stated in more general terms by Gamow *et al.*[32] This problem, which has come to be known as the "coding" problem, has been analyzed in terms of how the nucleotide sequence in nucleic acid determines the order of amino acids in polypeptide chains. Thus far it has not been possible to arrive at a unique solution of this problem.[32, 34] One aspect of this problem which has previously been alluded to only briefly is the role of the amino acid–transferring enzyme in determining the position of the amino acid on the RNA.[31] Particularly, we may ask whether the specificity of the activating enzyme is such that it will transfer the

activated amino acid only to a specific nucleotide sequence or configuration (or both) of the RNA. That such a specificity on the part of the enzyme may exist can be inferred from studies on the specificity of the acyl group acceptor in the case of fatty acid activation With these particular enzymes,[1, 3–6] CoA—but not other sulfhydryl compounds—is the fatty acid acceptor. Moreover, in acetate activation, pantetheine, a closely related derivative of CoA, is inactive as acyl acceptor.[35] If this specificity on the part of the amino acid–activating enzyme does exist, then the sequential arrangement of amino acids in protein may be determined both by the nucleotide sequence of the RNA and the specificity of the individual amino acid–activating enzymes. This distinction becomes important if we consider whether the homologous amino acid–activating enzymes from different sources have the same or different specificities with regard to the acceptor site on the RNA. The foregoing arguments pose the question of whether, in all species, a given amino acid is always associated with the same structural or sequential arrangement of nucleotides. We are presently investigating this question by studying the incorporation of a single amino acid onto RNA, using the appropriate amino acid–activating enzyme from different sources and with RNA isolated from various species.

## SUMMARY

An enzymatic system for linking amino acids to RNA has been described. The incorporation of a single amino acid requires the corresponding amino acid–activating enzyme, a specific RNA fraction, ATP, and $Mg^{++}$. Our experiments indicate that the amino acid–activating enzyme catalyzes both the formation of the adenyl amino acid and the transfer of the amino acid moiety to RNA. Experiments with several amino acids indicate that there are specific binding sites for these amino acids associated with the RNA.

* This work was supported by a grant from the United States Public Health Service. The following abbreviations have been used: ATP, adenosine triphosphate; AMP, adenosine-5′-monophosphate; CoA, coenzyme A; DNA, deoxyribonucleic acid; PP, inorganic pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; RNA, ribonucleic acid.

† Predoctoral Fellow of the National Science Foundation.

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## *SOME PROBLEMS CONCERNING THE ACTIVATION OF AMINO ACIDS*

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Any enzyme system that is proposed to account for the activation of amino acids prior to their conversion to proteins must have certain characteristics: (1) the reaction must involve all the naturally occurring amino acids; (2) there may reasonably be a correspondence between the degree of activation of a given amino acid and the frequency of occurrence of that amino acid in proteins; and (3) the proposed reaction should be present in all living cells.

Hoagland[1] discovered an amino acid-dependent exchange between inorganic pyrophosphate (PP) and ATP in extracts of rat liver that appears to reflect a system for the carboxyl activation of amino acids. DeMoss and Novelli[2] found this exchange reaction widely distributed in micro-organisms. Since the exchange reaction seemed to involve many of the naturally occurring amino acids and also because the reaction was found in partially resolved systems that catalyze the incorporation of amino acids into proteins, the amino acid-dependent PP-ATP exchange system has been implicated as the enzyme system responsible for the activation of amino acids for the biosynthesis of proteins.[3–5]

My purpose in this discussion is to report some observations on the activation of amino acids, as reflected by the PP-ATP exchange reaction, that are inconsistent with the idea that this system is responsible for the activation of amino acids for protein biosynthesis. These observations may be real or artifacts, but in either

case they must be adequately explained if we are to evaluate the function of such systems in the biosynthesis of proteins.

It has been indicated in previous papers in this symposium[6, 7] that the activation of amino acids is generally measured by one of two methods: (1) the amino acid-dependent exchange of inorganic pyrophosphate with ATP:

$$ATP + PP^{32} \overset{AA}{\rightleftharpoons} ATP^{32} + PP^{32}$$

and (2) the formation of amino acid hydroxamates when preparations are incubated with amino acids, ATP, and relatively high concentrations of hydroxylamine:

$$\begin{array}{l} H \\ | \\ R\text{-}C\text{-}COOH \\ | \\ NH_2 \end{array} + ATP + NH_2OH \rightarrow \begin{array}{l} H \\ | \\ R\text{-}C\text{-}CONHOH \\ | \\ NH_2 \end{array} + PP + AMP$$

I should like to examine briefly the sort of information obtained through the application of both these methods to the general problem of the activation of amino acids and to make some attempt to interpret the various findings.

*The Amino Acid-dependent PP-ATP Exchange.*—During studies on the activation of amino acids by crude extracts of *Escherichia coli*, DeMoss and Novelli[2] observed that only eight to ten (cysteine, methionine, leucine, isoleucine, valine, tyrosine, tryptophan, phenylalanine, histidine, and possibly serine) of the naturally occurring amino acids significantly *stimulated* the exchange of PP with ATP. The word "stimulated" is used advisedly, since, in crude extracts of all tissues, some PP ordinarily exchanges with ATP in the absence of added amino acids. Upon the addition of a complete mixture of amino acids or of certain individual amino acids, the rate of exchange of PP with ATP increases. This background exchange, in the absence of added amino acids, is troublesome in calculating the effect of individual amino acids. This endogenous exchange is caused partly, but not entirely, by the presence of amino acids in the crude extracts; it can be reduced but not completely eliminated by dialysis, by repeated reprecipitation of the protein with saturated ammonium sulfate, or by treatment of the extracts with charcoal or with a mixture of anion and cation exchange resins. The observation that not all the amino acids stimulated the PP-ATP exchange reaction in the *E. coli* preparation can be variously interpreted. One may assume that (*a*) the enzymes required for the exchange with the apparently inactive amino acids were destroyed during the preparation of the extracts; (*b*) these enzymes were already saturated with their respective amino acid and therefore the further addition of amino acid could not elicit additional response (this amounts to saying that the endogenous exchange is being catalyzed by the inactive amino acids that are already saturating their respective enzymes); (*c*) it could be assumed that the inactive amino acids are activated through a mechanism not involving a PP-ATP exchange.

In Table 1, data are presented that compare the stimulation of the PP-ATP exchange by individual amino acids in extracts of five micro-organisms, four animal tissues, and one plant tissue extract. In the extracts from the micro-organisms, rat liver, rat kidney, and peas (although there is some individual variation), all the

significantly active amino acids are among those mentioned previously. With extracts from guinea-pig tissues, on the other hand, there seems to be a small but significant exchange with almost all the amino acids. Although the exchange rates with mammalian tissues are only about one-tenth the rate in microbial extracts, the highest exchange rates with individual amino acids are seen largely with those amino acids that are active in micro-organisms. I think we can generalize these observations by saying that alanine, arginine, aspartic acid, asparagine, glutamic acid, glutamine, glycine, proline, hydroxyproline, and lysine have little or no ability to catalyze the PP-ATP exchange reaction. In this connection it is interesting that the combination of asparagine, aspartic acid, glutamine, and glutamic acid represents a large percentage of the total amino acid residues of a number of proteins (i.e., insulin, 26 per cent; egg albumin, 21 per cent; edestine, 28 per cent; and zein, 29 per cent).[8] If it should prove that these amino acids are not activated by a process involving a PP-ATP exchange, then some other means of activating these amino acids needs to be found if the activating system under discussion is to be implicated in protein synthesis. DeMoss and Novelli[2, 4] have suggested that the latter group of amino acids might be activated by an anhydride exchange reaction, but evidence in support of this suggestion has not been obtained.

TABLE 1

RATE OF THE PP-ATP EXCHANGE WITH INDIVIDUAL AMINO ACIDS IN VARIOUS EXTRACTS*

| | Pyrophosphate-Adenosine Triphosphate Exchange (μmoles/Hr/Mg of Protein) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Amino Acid | *E. coli* | *M. lactilyticus* | *N. crassa* | *A. aerogenes* | *A. fischeri* | Rat Liver | Rat Kidney | Guinea-Pig Pancreas | Guinea-Pig Liver | Green Peas |
| Complete AA | 7.30 | 6.40 | 1.67 | 21.0 | 12.4 | 0.37 | 0.23 | 1.20 | 0.99 | 0.85 |
| Alanine | 0 | 0 | 0.14 | 0.33 | 0.05 | 0 | 0 | 0.02 | 0.01 | 0 |
| Arginine | 0 | 0 | 0 | 0 | 0.12 | 0 | 0 | 0.05 | 0 | 0 |
| Aspartic acid | 0 | 0 | 0.14 | 0 | 0.19 | ... | 0.06 | 0 | 0 | 0 |
| Asparagine | 0 | 0 | 0.14 | 0 | 0.31 | ... | 0 | 0.11 | 0 | 0 |
| Glutamic acid | 0.07 | 0 | 0.14 | 0 | 0.37 | 0 | 0 | 0.02 | 0 | 0 |
| Glutamine | 0 | 0 | 0 | 0 | 0.37 | 0 | 0 | 0 | 0 | 0 |
| Glycine | 0 | 0 | 0 | 0 | 0.05 | 0 | 0 | 0.03 | 0.07 | 0 |
| Hydroxy-proline | 0 | 0.08 | 0 | 0 | 0 | 0 | 0 | 0 | 0.05 | 0 |
| Lysine | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.05 | 0 |
| Proline | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.08 | 0.03 | 0 |
| Serine | 0.33 | 0.41 | 0.36 | 0 | 0 | 0 | 0 | 0.29 | 0.05 | 0 |
| Threonine | 0 | 0 | 0.14 | 0 | 0 | 0 | 0 | 0.23 | 0.02 | 0 |
| Tryptophan | 0.36 | 0.98 | 0.14 | 0 | 0.20 | 0.04 | 0 | 0.06 | 0.06 | 0.12 |
| Histidine | 0.57 | 0 | 0.44 | 0 | 0 | 0.02 | 0 | 0.25 | 0.43 | 0 |
| Phenylalanine | 0.95 | 0 | 0.55 | 0.33 | 0 | 0.02 | 0.13 | 0 | 0.13 | 0 |
| Methionine | 2.53 | 0.34 | 0 | 2.02 | 2.34 | 0.12 | 0.02 | 0 | 0.55 | 0.02 |
| Tyrosine | 3.00 | 0.41 | 0.87 | 2.37 | 0.12 | 0.13 | 0.04 | 0.25 | 0.19 | 0.29 |
| Valine | 3.00 | 2.07 | 0.65 | 9.14 | 3.60 | 0.04 | 0 | 0.08 | 0.32 | 0.27 |
| Leucine | 3.46 | 3.34 | 1.59 | 10.04 | 5.02 | 0.15 | 0.37 | 0.20 | 0.30 | 0.30 |
| Isoleucine | 5.12 | 5.01 | 0.16 | 16.59 | 2.34 | 0.10 | 0.22 | 0.08 | 0.71 | 0.04 |
| Cysteine | ... | 1.59 | 0.55 | 4.73 | ... | 0.12 | 0.28 | 0.15 | 0.06 | 0.02 |

* Data on *E. coli*, *Micrococcus lactilyticus*, *Neurospora crassa*, and *Aerobacter aerogenes:* J. A. DeMoss, Ph.D. thesis, Dept. of Microbiology, Western Reserve University School of Medicine, 1957. Data on A. fischeri: M. J. Cormier and G. D. Novelli, unpublished observations. Data on peas: J. W. Davis and G. D. Novelli, *Arch. Biochem. and Biophys.* (in press).

*The Formation of Amino Acid Hydroxamates.*—It has been mentioned that amino acid carboxyl activation can also be measured through the formation of hydroxamic acid. This method has been used for the isolation and purification of several amino acid-activating enzymes.[9–12] In the hope of learning something about a possible activation of the "inactive" amino acids, we compared the rate of the PP-ATP ex-

TABLE 2

COMPARISON OF RATE OF PP-ATP EXCHANGE WITH HYDROXAMATE IN *Escherichia coli**

| AMINO ACID | RATE (μMOLES/HR/MG OF PROTEIN) | | EXCHANGE / HYDROXAMATE | AMINO ACID | RATE (μMOLES/HR/MG OF PROTEIN) | | EXCHANGE / HYDROXAMATE |
|---|---|---|---|---|---|---|---|
| | Exchange | Hydroxamate | | | Exchange | Hydroxamate | |
| Alanine | 0 | 0.008 | ... | Serine | 0.33 | 0.011 | 30 |
| Arginine | 0 | 0.019 | ... | Threonine | 0 | 0.013 | ... |
| Aspartic acid | 0 | 0.447 | ... | Tryptophan | 0.41 | 0.025 | 16 |
| Asparagine | 0 | 0.332 | ... | Histidine | 0.62 | 0.002 | 310 |
| Glutamic acid | 0 | 0.155 | ... | Phenylalanine | 0.98 | 0.032 | 30 |
| Glutamine | 0 | 0.207 | ... | Methionine | 2.50 | 0.021 | 120 |
| Glycine | 0 | 0.008 | ... | Tyrosine | 2.99 | 0.067 | 45 |
| Hydroxyproline | 0 | 0.004 | ... | Valine | 2.99 | 0.008 | 375 |
| Lysine | 0 | 0 | ... | Leucine | 3.44 | 0.003 | 1150 |
| Proline | 0 | 0.003 | ... | Isoleucine | 5.08 | 0.003 | 1690 |

* Data from J. A. DeMoss, Ph.D. thesis, Dept. of Microbiology, Western Reserve University School of Medicine, 1957.

TABLE 3

COMPARISON OF RATE OF PP-ATP EXCHANGE WITH HYDROXAMATE FORMATION IN *Achromobacter fisheri**

| AMINO ACID | RATE (μMOLES/HR/MG OF PROTEIN) | | EXCHANGE / HYDROXAMATE | AMINO ACID | RATE (μMOLES/HR/MG OF PROTEIN) | | EXCHANGE / HYDROXAMATE |
|---|---|---|---|---|---|---|---|
| | Exchange | Hydroxamate | | | Exchange | Hydroxamate | |
| Alanine | 0.05 | 0 | 0 | Serine | 0 | 0 | 0 |
| Arginine | 0.12 | 0 | 0 | Threonine | 0 | 0 | 0 |
| Aspartic acid | 0.19 | 0.43 | 0.44 | Tryptophan | 0.20 | 0 | 0 |
| Asparagine | 0.31 | 0.22 | 1.4 | Histidine | 0 | 0.02 | ... |
| Glutamic acid | 0.37 | 0.04 | 9.3 | Phenylalanine | 0 | 0 | 0 |
| Glutamine | 0.37 | 0.14 | 2.7 | Methionine | 2.34 | 0.01 | 2.34 |
| Glycine | 0.05 | 0.09 | 0.55 | Tyrosine | 0.12 | 0.07 | 1.7 |
| Hydroxyproline | 0 | 0 | 0 | Valine | 3.60 | 0 | 0 |
| Lysine | 0 | 0 | 0 | Leucine | 0 | 0 | 0 |
| Proline | 0 | 0 | 0 | Isoleucine | 2.34 | 0 | 0 |

* M. J. Cromier and G. D. Novelli, unpublished data.

TABLE 4

COMPARISON OF RATE OF PP-ATP EXCHANGE WITH HYDROXAMATE FORMATION IN GUINEA-PIG LIVER*

| AMINO ACID | RATE (μMOLES/HR/MG OF PROTEIN) | | EXCHANGE / HYDROXAMATE | AMINO ACID | RATE (μMOLES/HR/MG OF PROTEIN) | | EXCHANGE / HYDROXAMATE |
|---|---|---|---|---|---|---|---|
| | Exchange | Hydroxamate | | | Exchange | Hydroxamate | |
| Alanine | 0.01 | 0 | 0 | Serine | 0.05 | 0.031 | 1.6 |
| Arginine | 0 | 0 | 0 | Threonine | 0.02 | 0 | 0 |
| Aspartic acid | 0 | 0.014 | ... | Tryptophan | 0.06 | 0.037 | 1.6 |
| Asparagine | 0 | 0.20 | ... | Histidine | 0.43 | 0.029 | 14.8 |
| Glutamic acid | 0 | 0.112 | ... | Phenylalanine | 0.13 | 0.014 | 9.3 |
| Glutamine | 0 | 0.112 | ... | Methionine | 0.55 | 0.015 | 36.6 |
| Glycine | 0.07 | 0.029 | 2.42 | Tyrosine | 0.19 | 0.025 | 7.6 |
| Hydroxyproline | 0.05 | 0.019 | 2.6 | Valine | 0.32 | 0.015 | 21.3 |
| Lysine | 0.05 | 0.029 | 1.7 | Leucine | 0.30 | 0.014 | 21.5 |
| Proline | 0.03 | 0.027 | 1.1 | Isoleucine | 0.71 | 0.014 | 50.3 |
| | | | | Cysteine | 0.06 | 0.019 | 3.2 |

* G. D. Novelli and A. N. Best, unpublished data.

change reaction with the rate of hydroxamic acid formation, as catalyzed by the individual amino acids, in several different extracts. These data are presented in Tables 2, 3, and 4. The points of significance are (1) the amino acids can be grouped into three classes: (*a*) those that catalyze only the formation of hydroxamic acid,

(*b*) those that catalyze only the exchange, and (*c*) those that catalyze both reactions; (2) with the amino acids that catalyze both reactions there seems to be no definite relation between the rates of the two processes. Although it is true that with the tryptophan-activating enzyme from beef pancreas[9] and the tyrosine-activating enzyme from hog pancreas[10] there is a definite relation between the PP-ATP exchange and hydroxamic acid formation, the data in Tables 2, 3, and 4 suggest that, in general, the two methods may be measuring quite different reactions in the mechanism of activation of the carboxyl group. Evidence in support of this suggestion has been obtained by M. J. Cormier and Novelli (unpublished). We elected to purify the glycine-activating enzyme from *Achromobacter fisheri* which in a survey experiment seemed to catalyze hydroxamic acid formation without catalyzing the PP-ATP exchange. The sixteen fold purified enzyme apparently activates glycine by a process involving the elimination from ATP of one molecule of orthophosphate. Its characteristics are rather similar to those of glutamine synthetase.[13] Thus it is probably unsafe to draw general conclusions about amino acid activation from observations made through the use of either method of measurement.

*The Mechanism of the PP-ATP Exchange Reaction.*—By analogy with the proposal of Berg on the activation of acetate,[14] Hoagland *et al.*[3] proposed that the PP-ATP exchange reaction occurred through the intermediate formation of an enzyme-bound aminoacyl adenylate. Support for this formulation was provided by DeMoss, Genuth, and Novelli,[15] who synthesized leucyl adenylate and showed that it was rapidly converted to ATP in the presence of PP by an enzyme from *E. coli* that carried out a leucine-dependent PP-ATP exchange. These experiments suggested that such aminoacyl adenylates might indeed be intermediates in the exchange reactions and might represent the activated amino acid. However, since evidence for the formation of such compounds during the exchange reaction has not appeared, one cannot say with certainty that such compounds are true intermediates. Indeed, later findings have cast some doubt on the exact role of the aminoacyl adenylates in the exchange reaction. Berg[16] has reported that a methionine-activating enzyme isolated from yeast that is specific for methionine in the PP-ATP exchange reaction could nevertheless convert a variety of synthetic aminoacyl adenylates to ATP when incubated with PP. This suggested a great lack of specificity in the backward reaction. This observation was at variance with our earlier report that the leucine-activating enzyme from *E. coli* exhibited the same specificity in ATP synthesis from aminoacyl adenylates as it had shown in the PP-ATP exchange reaction. To explore this problem further, we tested the ability of the pancreatic tryptophan-activating enzyme[9] to form ATP from a variety of synthetic aminoacyl adenylates at various stages in the purification of the enzyme. These results are presented in Table 5. It seems clear that even the 145 fold purified enzyme (Am 65) can convert a large variety of synthetic aminoacyl adenylates to ATP. This lack of specificity extends to D compounds as well as to L,S-methylcysteinyl adenylate. Since ATP is not formed from carbobenzoxyleucyl adenylate or from acetyl adenylate, a free amino group in the compound seems to be necessary for activity. It does not seem reasonable that an enzyme so highly specific in one direction would be so non-specific in the backward reaction if these aminoacyl adenylates were the true intermediate in the exchange reaction. One possible explanation for the apparent lack of specificity for ATP synthesis with the synthetic

compounds might be that both the methionine-activating enzyme and the tryptophan-activating enzyme, although considerably purified, are apparently still contaminated with a bound form of their respective amino acid, as judged by a highly significant exchange rate in the absence of added amino acid. It thus becomes possible that an anhydride exchange between the bound amino acid and the amino acid of the synthetic adenylate may occur to make the correct compound on the enzyme surface. Thus the specificity would be maintained. We are attempting to obtain experimental evidence to rationalize this point, but it is difficult to eliminate the last traces of bound amino acid from the enzyme.

TABLE 5*

PRODUCTION OF ATP FROM AMINO ACID ADENYLATES BY THE TRYPTOPHAN-ACTIVATING ENZYME† AT VARIOUS STAGES OF PURIFICATION‡

| ADENYLATE | μMOLES OF ATP FORMED IN 15 MINUTES | | |
|---|---|---|---|
| | Sup II | Ac I | Am 65 |
| D-Alanine | 0.083 | 0.125 | 0.111 |
| L-Alanine | 0.606 | 0.550 | 0.94 |
| D-Valine | 0.570 | 0.525 | 1.04 |
| L-Valine | 0.690 | 0.971 | 1.195 |
| D-Methionine | 1.64 | 1.18 | 1.76 |
| L-Methionine | 1.18 | 1.76 | 1.23 |
| D-Phenylalanine | 1.40 | 1.40 | 1.44 |
| L-Phenylalanine | 1.67 | 1.20 | 1.68 |
| D-Tryptophan | ... | ... | 0.75 |
| L-Tryptophan | 1.34 | 1.02 | 1.08 |
| L,S-Methylcysteine | 0.558 | 0.39 | 0.59 |
| L-Leucine | 1.002 | 0.945 | 1.078 |
| Carbobenzoxy-L-leucine | 0 | ... | ... |
| Acetyl | 0 | ... | ... |

* In each case 5 μmoles of compound were incubated with 60 hydroxamate units of enzyme, 50 μmoles of KF, 10 μmoles of $PP^{32}$ in $PO_4$ buffer, pH 6.8.
† See n. 9.
‡ R. W. McRorie and G. D. Novelli, unpublished data.

Support for the aminoacyl adenylates as intermediates in the activation reaction is provided by experiments with $O^{18}$-labeled amino acids as detailed in another paper in this symposium (Boyer).[17] These experiments, however, are not decisive, since they indicate only that a physical union has occurred between the carboxyl group of the amino acid and the adenylic acid moiety of ATP, without indicating the exact chemical structure of the intermediate.

Although circumstantial evidence is mounting in support of the idea that amino acid activation as reflected by the PP-ATP exchange reaction is involved in protein biosynthesis, the present observations suggest that more data are necessary before a definitive statement can be made.

* Operated by Union Carbide Nuclear Company for the U.S. Atomic Energy Commission.

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## *TRACING OF THE IN VIVO PATH FROM AMINO ACID TO PROTEIN**

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The problem of protein synthesis has many facets. The previous speakers on this symposium have outlined and discussed the excellent researches with cellular fractions and partially purified enzyme systems which demonstrate activations and incorporations of amino acids dependent upon energy derived from cleavage of ATP. Information from these studies suggests that similar mechanisms operate in vivo and thus that, for every peptide bond formed, at least one ATP molecule is split. Over the past several years, studies have been made in our laboratory on the mechanisms of ATP utilization and formation in various reactions catalyzed by cell fractions and purified enzymes. An important part of these researches has dealt with the fate of the oxygen atoms in substrate molecules participating in the coupled reactions as studied by use of the heavy isotope of oxygen, $O^{18}$. These and related studies by other investigators have yielded fundamental information about the course of the reactions and have suggested, as will be outlined presently, that it might be possible to put to test the hypothesis that protein synthesis in the intact cell involves cleavage of an ATP molecule for each peptide bond formed, through participation of an intermediate formed by combination of part of the ATP molecule with the carboxyl group of the amino acid. The purpose of this contribution is to present results of studies on the fate of the carboxyl oxygens of amino acids, which allow tracing the path of amino acids to protein in an intact cell, and to deduce some aspects of the nature of the primary reactions and other reactions associated with the protein synthesis.

When an ATP molecule is cleaved to ADP and iP (inorganic orthophosphate), or to ADP and PP (inorganic pyrophosphate), an oxygen atom from some source must be incorporated into the products. In some metabolic processes, such as muscular contraction, hydrolysis of ATP appears to be involved, and the oxygen would be furnished by water. In syntheses coupled to ATP degradation, the possibility exists that the required oxygen would be furnished by one of the substrates participating in the over-all reactions. That such is indeed the case is shown by the researches summarized in Table 1. This table gives the results of $O^{18}$ studies

with various systems of the general reaction types as follows:

$$ATP + ROH + R'H \begin{cases} \nearrow R\text{-}R' + ADP + iP \\ \searrow R\text{-}R' + AMP + PP \end{cases}$$

TABLE 1

DIRECT O TRANSFERS IN SYNTHESES COUPLED TO ATP CLEAVAGE

ATP + R-O$^{18}$H → PRODUCT

| | |
|---|---|
| iP-O$^{18}$ (+ ADP) | Glutamate → Glutamine* |
| | Succinate → Succinyl CoA† |
| | $CO_2$ + Ornithine → Citrulline‡ |
| | 3-Phosphoglycerate → Acyl Enzyme§ |
| | Inosine-5-Phosphate → AMP‖ |
| | (GTP Cleavage) |
| AMP-O$^{18}$ (+ PP) | Fatty Acid → Acyl CoA* |
| | Pantoate → Pantothenate# |
| | Xanthosine-5-Phosphate → Guanosine-5-Phosphate** |
| | Amino Acid → Acyl Hydroxamate†† |
| | Amino Acid → Protein (?) |
| PP-O$^{18}$ (+ AMP) | None Observed |
| ADP-O$^{18}$ (+ iP) | None Observed |

* P. D. Boyer, O. J. Koeppe, and W. W. Luchsinger, *J. Am. Chem. Soc.*, **78**, 356, 1956; A. Kowalsky, C. Wyttenbach, L. Langer, and D. E. Koshland, Jr., *J. Biol. Chem.*, **219**, 719, 1956.

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In every such reaction that has been studied, oxygen from a substrate appears in the products from ATP cleavage; indeed, the results may warrant a generalization that reactions of this type are accompanied by transfer of oxygen from substrates to a phosphate compound derived from the nucleotide cleaved. Only two types of transfer have thus far been noted—that in which the oxygen appears in the iP formed and that in which the oxygen appears in the AMP. It seems likely that in the over-all reactions demonstrated by Kornberg *et al.*[1] for formation of 5-phosphoribosylpyrophosphate from ATP and ribose-5-phosphate, followed by transfer of the 5-phosphoribosyl moiety to adenine, with liberation of pyrophosphate, oxygen from the C-1 of the ribose-5-phosphate would appear in the pyrophosphate. There are at present no observed or indicated reactions with transfer of oxygen to ADP.

The most recent addition to the listing in Table 1 is the coupled reaction of amino acid activation, with formation of amino acid hydroxamates; the study of this reaction was a collaborative undertaking of our group and that of Professors Lipmann and Zamecnik at Harvard.[2] Results of these studies will be presented in slightly more detail to indicate the nature of the experimental procedures and findings. Essential final experimental data are given in Table 2. Tryptophan labeled in the carboxyl group with O$^{18}$ was incubated with the highly purified tryptophan-activating enzyme from pancreas[3] until conversion of the added ATP in the stoichiometric

reaction was essentially complete. The AMP and PP formed were separated, and iP formed therefrom by the action of alkaline phosphatase on the AMP and by acid hydrolysis of the PP. The iP was isolated as $KH_2PO_4$, heated to form water, the water equilibrated to exchange its oxygen atoms with $CO_2$, and the ratio of mass 46 to mass 44 of the $CO_2$ was measured in the mass spectrometer. The data showed that in the control sample where non-labeled tryptophan was used, the oxygens from PP and AMP had the normal isotopic content. With the labeled tryptophan, within experimental error, one oxygen from the tryptophan carboxyl appeared in the phosphate of the AMP for each tryptophan hydroxamate formed. These results, together with the demonstrations that amino acyl adenylates are active in such enzyme systems,[4, 5] strongly suggest that the reaction involves an intermediate in which the amino acid is linked through a carboxyl oxygen to the phosphorus of AMP, and that this intermediate is the amino acyl adenylate or a closely related structure that may exist only in combination with the enzyme.

TABLE 2

DIRECT OXYGEN TRANSFER IN AMINO ACID ACTIVATION

$$ATP + RCOOH + NH_2OH \rightarrow AMP + PP + R{-}\overset{\overset{O}{\|}}{C}{-}NHOH$$

| Tryptophan Used | Atom Per Cent Excess $O^{18}$ Found in Phosphate Oxygen | |
|---|---|---|
| | AMP × 4 | Pyrophosphate × 8 |
| 0.00 | 0.0 | 0.0 |
| 1.09 | 0.99 | 0.0 |

The studies with intact cells were initiated soon after the finding in our laboratory[6] that direct oxygen transfer occurred in the synthesis of glutamine and acetyl coenzyme A, and they received further impetus by the reports on amino acid activation in cell-free systems.[7, 8] The studies were based on the supposition that if similar reactions occurred in protein synthesis by intact cells, the transfer of one oxygen from the amino acid carboxyl to phosphate compounds should occur for each amino acid that found its way into protein. For the studies, it appeared necessary to have a cell which could not synthesize its own amino acids and would therefore be dependent upon any labeled amino acids added and, in addition, would not be likely to catalyze the loss of the excess $O^{18}$ from the carboxyl group by reactions other than those of protein synthesis. Further, the cell should not possess reactions which cause a rapid exchange of phosphate oxygen with water oxygen. In particular, the cell should lack the capacity for oxidative phosphorylation coupled to $O_2$ uptake because a very rapid exchange of phosphate oxygen with water oxygen has been found to be closely associated with the oxidative phosphorylation process.[9] A bacterial species which appeared to meet these requirements was *Leuconostoc mesenteroides* P-60. In addition, use of this organism was advantageous because its sole source of energy is derivable from the stoichiometric breakdown of glucose to lactic acid, ethanol, and carbon dioxide. Conventional media for this organism were further simplified, and, for the experiments to be reported, the organism was grown on a medium containing 0.002 *M* glucose, 0.0007 *M* $KH_2PO_4$, 0.16 *M* total amino acids (as casein, hydrolyzed by acid in $H_2O$ or $H_2O^{18}$ and freed of iP, with added tryptophan) together with small amounts of salts, purines, pyrimidines, and vitamins.

Preliminary studies with use of $H_2O^{18}$ or with iP-$O^{18}$ showed that this organism catalyzed a slow but detectable exchange of the oxygens of iP with those of water; fortunately, this exchange appeared sufficiently limited to allow the contemplated experiments. The organism was thus grown on a medium containing carboxyl-$O^{18}$-labeled amino acids, and the medium iP and the cellular iP + labile P fractions were isolated and analyzed for $O^{18}$ content. Somewhat to our surprise, only traces of $O^{18}$ were detectable in these fractions, and the amount present was far less than that which must have been lost from the amino acids. Localized transfer of the oxygen to another substance seemed probable, and lactate appeared to be a likely candidate. Previous studies in our laboratory[10] have shown that in glycolysis, through the intermediate formation of 1,3-diphosphoglycerate, an oxygen of inorganic phosphate appears in the carboxyl group of 3-phosphoglycerate. The carboxyl of 3-phosphoglycerate in subsequent reactions appears as the carboxyl of lactate.

TABLE 3

ATOM PER CENT EXCESS $O^{18}$ IN FRACTIONS AFTER CELL GROWTH

| Exp. | Medium iP | Cellular iP and Labile P | Protein-Exchangeable Oxygen* | Lactate Carboxyl |
|---|---|---|---|---|
| 1 | <0.01 | 0.015 | | |
| 2 | <0.02 | 0.014 | 0.51 | 0.025 |
| 3 | <0.01 | 0.015 | 0.49 | 0.022 |

* Assuming equal distribution of $O^{18}$ in all exchangeable oxygens; original amino acids = 0.70 atom per cent excess.

A more complete analysis for the fate of the amino acid carboxyl oxygen was thus made. The results of some experiments are shown in Table 3, which gives the atom per cent excess $O^{18}$ in various fractions. The remaining iP of the medium, to which carrier phosphate had to be added for isolation and analysis, showed no detectable excess $O^{18}$. The small amount of excess $O^{18}$ in the cellular iP and labile P fractions is apparent. In order to approximate the amount of $O^{18}$ originally present in the amino acid carboxyl groups and that which remained in the peptide carbonyl groups, determinations were made of the amount of $O^{18}$ in the oxygens of the hydrolyzed casein used and of the isolated bacterial protein fraction which were readily exchangeable with oxygens of water when heated under strongly acidic conditions. The exchangeable oxygen fraction comprises the amino acid carboxyls of the hydrolyzed proteins and the terminal carboxyls of the aspartic and glutamic acid residues. It is not known to what extent the various components which make up the exchangeable pool were labeled, except that the amino acid carboxyl groups must have contained at least 0.55 atom per cent excess $O^{18}$, half the amount present in the $H_2O^{18}$ used for casein hydrolysis The figures given in the table are based on the assumption that all the exchangeable oxygens were equally labeled. By considering various possible extremes of distribution of the $O^{18}$, it may be concluded that the peptide carbonyl groups contain at least half and probably nearly as much excess $O^{18}$ as the carboxyl groups of the original amino acids. For determination of the excess $O^{18}$ in the lactate, the lactate was isolated as the pure silver salt, decarboxylated as such, and the mass 46/44 ratio of the $CO_2$ determined. No dilution was necessary, and the atom per cent excess reported, although not large, is a fairly accurate figure.

In conjunction with one of these experiments, determinations were also made of

the excess $O^{18}$ present in the acid-stable phosphate fraction of the bacterial cell; this represents principally phosphate from hydrolysis of polyribonucleotide. No excess $O^{18}$ was detected. The limits of the analyses and experiments were such to allow the approximation than not more than 1 out of 10, if any, of the phosphate groups had acquired an oxygen from the amino acid carboxyl. The amino acids had thus left no detectable trace on the polynucleotide phosphate; however, it should be recalled that transfer of an amino acyl group to a phosphate and removal as such would not result in the leaving of any oxygen of the amino acid with the phosphate.

The approximate distribution of the total excess $O^{18}$ lost from the amino acids incorporated into cellular protein is shown in Table 4. The medium iP, together with the cellular iP and labile P fractions, account for less than 1 per cent of the excess $O^{18}$. If equal distribution of the exchangeable oxygens of the amino acids is assumed, then about 36 per cent of the original total excess $O^{18}$ remained with the amino acid residues of the protein, and about 65 per cent had been transferred to the large lactate pool. For estimation of the amount in the protein-exchangeable oxygen fraction, the average residue weight of the amino acids of the isolated bacterial protein was determined by the amount of $\alpha$-amino acid carboxyl present in an appropriate hydrolyzate. The relative distribution of the total $O^{18}$ in the protein exchangeable oxygen and in the lactate carboxyl fractions is uncertain because, as mentioned previously, assignment of the $O^{18}$ found in the exchangeable oxygens among various $\alpha$-carboxyl and side-chain carboxyl groups is uncertain. Calculation of the total recovery of $O^{18}$ lost from the amino acids is fortunately relatively independent of the distribution of the $O^{18}$ in the exchangeable oxygens. Definitely over 90 per cent of the oxygen lost from the amino acids is recoverable in the lactate carboxyl group.

TABLE 4

DISTRIBUTION OF $O^{18}$ FROM AMINO ACIDS CELL GROWTH (IN PER CENT)

| Exp. | Medium iP | Cellular iP and Labile P | Protein-Exchangeable Oxygen* | Lactate Carboxyl | Total Recovery |
|---|---|---|---|---|---|
| 1 | <0.5 | <0.3 | 37 | 69 | 106 |
| 2 | <0.2 | <0.2 | 35 | 62 | 97 |

* Assuming equal distribution of $O^{18}$ in all exchangeable oxygens.

These findings establish that in vivo, in this bacterial species, as depicted in Figure 1, reactions by which amino acids are made into protein take place in such a manner that oxygen which must be lost from the amino acid carboxyl appears nearly quantitatively in the carboxyl groups of lactate. The only known, and indeed at present the only plausible, way in which this might occur is first by transfer of the oxygen from the amino acid to phosphate fractions, intermixing of the oxygens among these phosphate fractions to give iP labeled with $O^{18}$, transfer of the oxygen from the iP to the carboxyl of 3-phosphoglycerate by the glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase reactions, and subsequent conversion of the 3-phosphoglycerate to lactate. All this occurs with an exceptionally small amount of the excess $O^{18}$ appearing in the medium iP or the cellular iP and labile P fractions. By analogy, one might say that this is an example of highly organized teamwork, with the team passing the ball, $O^{18}$, a number of

times without dropping it and without letting it mix with a large number of similar balls present on the side lines and, indeed, right in the playing field.

The most important aspect of the data is the very strong support they give to the concept that in in vivo protein synthesis, each amino acid is activated through formation of an anhydride-type compound with a phosphate, where the amino acid carboxyl forms the bridge oxygen between the acyl carbon and the phosphorus. In addition, the data have important implications for other reactions associated with protein synthesis. The remarkable localization of the reactions within the cell and the lack of interchange of the phosphate compounds in this localized area

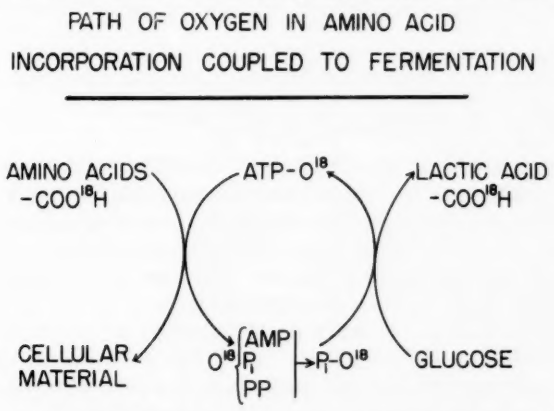


FIG. 1.—The path of the carboxyl oxygen of amino acids incorporated into protein by *Leuconostoc mesenteroides P-60*.

with other intracellular phosphate and with iP of the medium have already been mentioned. The high percentage of excess $O^{18}$ which remains with the amino acid residues in the protein suggests that, in the transfer of the amino acids across membranes, no reactions resulting in oxygen loss from the carboxyl occur and, further, that the extent of hydrolysis and resynthesis of cellular protein is limited. The tracer studies with the intact cell correlate well with in vitro studies using cellular fractions and establish applicability of projected reactions to cellular protein synthesis.

* The work presented was largely taken from the Ph.D. thesis of Dr. Stulberg. Researches reported were supported in part by grant AT-(11-1)341 of the AEC, by grant 4930 of the USPHS, and by the Hill Family Foundation.

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# *APPLICATION OF A THEORY OF ENZYME SPECIFICITY TO PROTEIN SYNTHESIS**

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The necessity for "activation" of an amino acid to allow it to form a peptide bond has two aspects. The first of these is thermodynamic and arises from the fact that the equilibrium in the reaction shown in equation (1) lies far to the left:

$$^{+}H_3N{-}CHRCOO^{-} + \overset{+}{H_3}NCHR'COO^{-} \rightarrow \overset{+}{H_3}N{-}CHRCO{-}NHC\overset{-}{O}O + H_2O \quad (1)$$

The suggestion of Lipmann[1] that the energy required to drive this reaction to the right came from adenosine triphosphate has been supported by the extensive work[2] which has been discussed by the previous speakers on this symposium. While the detailed mechanism of this energy transfer has not been fully elucidated, the ATP apparently forms an amino acid anhydride. The fact that an anhydride of relatively high-energy content is the method of driving the reaction thermodynamically fits in with organic practice in which acyl anhydrides are the usual reagents for acylations.

In addition to thermodynamic activation, there is a needed "kinetic activation." For example, the acyl phosphates and acyl adenylates acylate amines non-enzymatically at appreciable rates in aqueous solution,[3] but this reaction is certainly not rapid enough or selective enough to account for protein synthesis. The reaction of these acyl anhydrides to form peptides must be catalyzed, and it is this catalysis which is the subject of this paper.

An analysis of why this linking of amino acids presents such formidable difficulties is revealing. The difficulty appears to be caused by a combination of requirements, each of which, taken singly, is rather easily satisfied. The first requirement is that an individual position in the protein be occupied by one and only one amino acid. This by itself is not a difficult condition to satisfy, since enzymatic reactions of equally high specificity are well known. The second requirement is that a high-molecular-weight polymer be produced. Again, this, by itself, is not a unique condition, since the enzymatic formation of high-molecular-weight molecules from a given monomer is also familiar, e.g., carbohydrate polymerization catalyzed by phosphorylases. Finally, the macromolecule is formed from many different monomer units, but this requirement is also easily achieved if the units are randomly arranged, e.g., polynucleotide formation by polynucleotide phosphorylase.[4] However, the combined requirements, i.e., the synthesis of a macromolecule from many individual monomers to give a single specified sequence, is a problem of different magnitude. A mechanism involving an individual enzyme for each bond would be acceptable from the specificity point of view, but it would require an inconceivably large number of enzymes to form all the proteins of the cell. Moreover, there are other observed conditions, e.g., the necessity of feeding all the amino acids

within a short period of time[5] and the lack of intermediates,[6] which argue against a sequence of free enzymatic reactions like those in carbohydrate metabolism.

The best framework for explaining at least part of these phenomena has come from a mechanical analogy, i.e., the template. This analogy has been refined over the years, and recently Dalgleish has proposed an ingenious modification[7] which is able to account for both asymmetric labeling of a protein[8] and a rapid rate of synthesis. He suggests that the part of the peptide chain which has been completed peels off the template as it is formed, allowing new peptide chains to be started before the initial one is completed. Thus the time for formation of an individual protein molecule is extended, while the high number of molecules formed per unit time is maintained.

The deficiency of any template model is simply that it is only a mechanical analogy and, since we are making chemicals and not automobiles, that it has to be translated into chemical terms. For example, in the model of Dalgleish it is assumed that the completed peptide peels off the template; but why individual amino acids which were previously tightly adsorbed should suddenly prefer to be desorbed must await chemical reasoning. The first step in this reasoning is an attempt to deduce the nature of the template itself.

The evidence seems overwhelming that the template has the characteristics of an enzyme, since the three features cited above are observed singly in enzyme-catalyzed reactions. Certainly, inductive effects or van der Waals attractions would not account for the exclusive selection of isoleucine in competition with valine or serine in competition with threonine at a specific position in a peptide chain. Whether these interactions are provided by a protein or by a nucleic acid or by a combination of the two cannot be decided as yet, but the monodisperse nature of the peptide chains suggests that the thermodynamically activated amino acid anhydride is kinetically activated by a catalyst which behaves like, even if it is not identical with, an enzyme.

Let us, therefore, first examine the manner in which an enzyme exerts its specificity. It is apparent that this specificity does not arise from inductive effects in the substrate molecules. A change from a hydroxy to a methoxy group at the C-3 position of glucose will have negligible electrostatic effects on the basic or acidic properties of the C-6 hydroxyl, and yet this change is enough to block completely the action of hexokinase.[9] To explain this specificity, a "key-lock theory" was proposed.[10, 11] In essence this theory said that the enzyme was a rather rigid negative of the substrate and that the substrate had to fit into this negative to react. Hence a modification which had minor electrostatic effects but which would so increase the bulk or change the shape of the molecule that it could not fit on the enzyme surface would prevent reaction. This explanation is supported by the demonstration of steric repulsions in organic reactions and by the fact that the kinetics of enzyme action are compatible with an enzyme-substrate complex. It is capable of explaining almost all the observed specificity patterns.

On close examination,[12] however, it is clear that this hypothesis does not explain all cases, and, as usual, it is the anomalies which lead to revisions of our theories. The anomalies fall, in general, in the class that smaller analogous compounds react extremely slowly or not at all. For example, ribose-5-phosphate is hydrolyzed by 5′-nucleotidase much less rapidly than adenylic acid.[13] Since the ribose-5-phosphate

is simply adenylic acid without the purine, it could be argued that an attractive group is eliminated and hence the affinity to the enzyme is less (which it probably is). However, when this lower affinity is compensated for by comparing the velocities at enzyme saturation, the ribose-5-phosphate still reacts at $^1/_{100}$ the rate of adenylic acid. The key-lock theory would certainly predict equal rates at saturation levels. Similarly, the failure of alpha methyl glucoside to act as a substrate for amylomaltase[14] is inconsistent with this hypothesis. Alpha methyl glucoside has the same structure as the natural substrate maltose, except that it is smaller, i.e., two hydrogens on the aglycon carbon take the place of the second pyranose ring. As would be expected from its smaller, but otherwise similar, structure, alpha methyl glucoside should be absorbed on the enzyme surface. That it does is shown by its action as a competitive inhibitor. However, it is not hydrolyzed by the enzyme. In this case certainly, lack of access to the enzyme surface cannot explain its failure to be a substrate. Numerous similar anomalies can be found.[12]

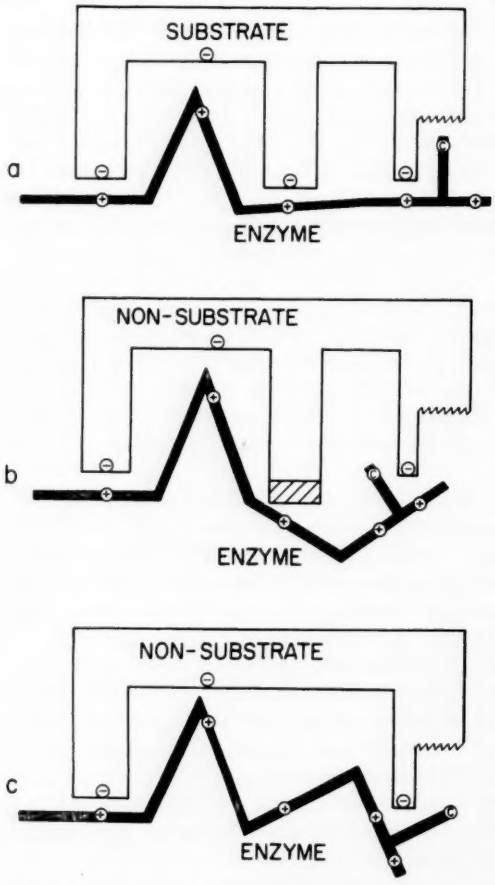


FIG. 1.—Interaction of enzyme with (*a*) substrate, (*b*) compound too large to be substrate, and (*c*) compound too small to be substrate. Circled pluses and minuses indicate any mutually attractive groups on enzyme and substrate. Circled *C* stands for catalytic group, and jagged line for bond to be broken.

The explanation that we suggest to explain these phenomena is as follows: (*a*) a precise orientation of catalytic groups is required for enzyme action; (*b*) the substrate may cause an appreciable change in the three-dimensional relationship of the amino acids at the active site; and (*c*) the changes in protein structure caused by a substrate will bring the catalytic groups into the proper orientation for reaction, whereas a non-substrate will not. This set of postulates has been called "the induced fit" theory for brevity and to emphasize that, while the idea of a fit is retained from the key-lock theory, the fit in this case occurs *only after* the changes induced by the substrate itself.

A simplified schematic representation is shown in Fig-

ure 1. In Figure 1, *a*, a catalytic group, *C*, is aligned with the bond to be broken (shown by the jagged line). In Figure 1, *b*, increasing the size of a group well removed from the bond to be broken destroys the alignment; and in Figure 1, *c*, the complete removal of the same group likewise destroys the alignment. Thus this theory explains why a substrate can be converted to a non-substrate by a decrease in, as well as by an increase in, its size.

Furthermore, there is independent support for the various postulates. First, the flexible nature of portions, if not all, of the protein chain is adduced from many sources, and these changes can be caused by small molecules, charged or uncharged. A typical example is the reversible denaturation of a number of enzymes by urea.[15] This denaturation is accompanied by changes in viscosity, optical rotation, and sedimentation constant, which certainly are indicative of an alteration in the geometry of the amino acids of the protein relative to each other. The urea undoubtedly causes these changes by competing for the internal hydrogen bonds of the protein.[16] The reversible nature of this denaturation shows that, upon removal of urea, the protein returns to its natural conformation. The urea, because of its particular structure, is attracted non-specifically to many peptide links, whereas a hydrogen-bonded substrate is attracted to only one or a small number of sites. It is certainly reasonable, however, to expect that the specifically attracted substrate will cause similar conformation changes in its immediate environment. Charged or hydrocarbon-type compounds are observed to cause analogous alterations in protein structure.

Second, there is support for the postulate that a precise orientation of catalytic groups is important in enzyme catalysis. The evidence comes from analogies to both heterogeneous and homogeneous catalysis in simple chemical systems and also from the observed behavior of the enzymes themselves.[17] Finally, the idea that the substrate is necessary to promote the proper orientation of catalytic groups can explain phenomena other than their specificity, for example, substrate-promoted isotopic exchanges.[18]

Provocative suggestions for translating the template into chemical terms can be derived from the induced-fit theory of specificity. For example, Figure 2 shows a possible sequence of reactions in the formation of a peptide bond in the center of a protein molecule. In this illustration, each activated amino acid can form an acyl bond with its appropriate position on the synthesizing template. However, we again assume that this template is not initially in its proper orientation but that this orientation is induced by the presence of the previous amino acid. Thus in Figure 2, *a*, the tenth amino acid has formed an acyl bond and hence has brought position 11 into the proper alignment (schematically indicated by being on a horizontal straight line) to attack the acyl group of the amino acid anhydride,

$$\begin{array}{c} \mathrm{O} \\ \| \\ \mathrm{X{-}C{-}CHR_{11}{-}NH_3^+}. \end{array}$$

After formation of the acyl-template bond (Fig. 2, *b*), the amino group is in the appropriate position to attack the carbonyl carbon of the tenth amino acid. As this peptide bond is formed (Fig. 2, *c*), the completed portion of the peptide peels off, and position 12, which was previously unreactive, is brought into proper orienta-

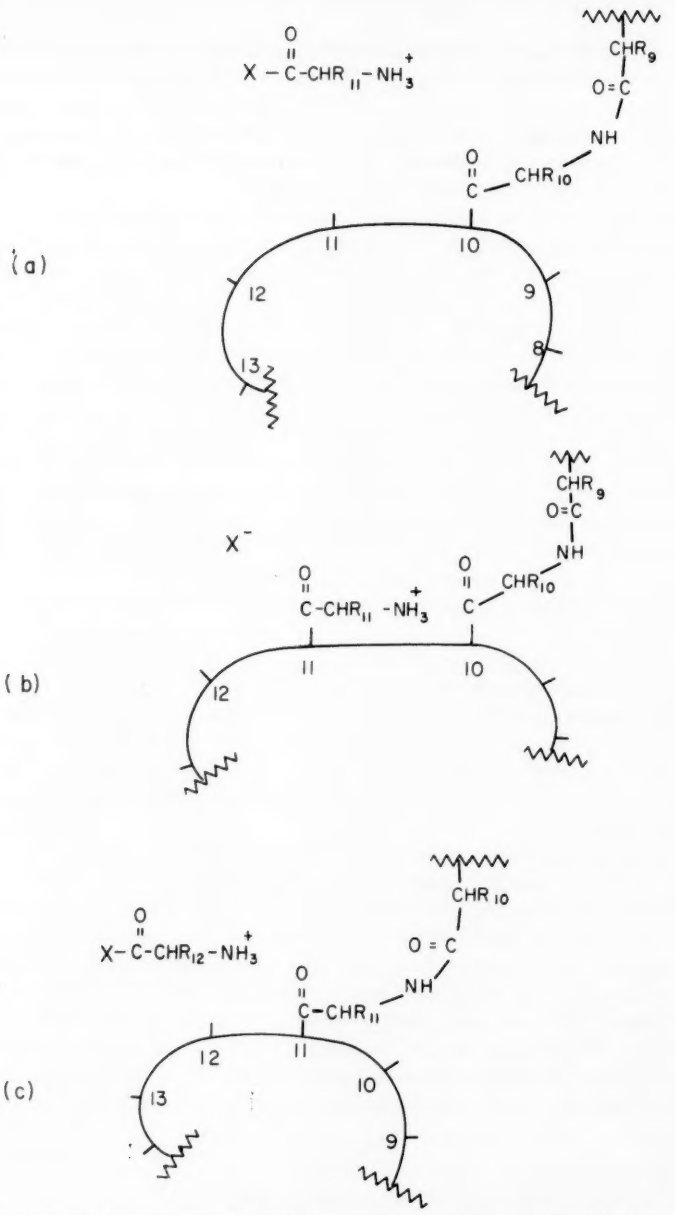


FIG. 2.—Possible sequence of events in protein synthesis. (*a*) eleventh activated amino acid approaches template, in which eleventh site has been "induced" into reactive position; (*b*) acyl-template bond is formed, and favorable geometry for reaction between eleventh and tenth amino acids established; (*c*) formation of peptide bond between eleventh and tenth amino acids induces site 12 into reactive position and allows completed portion of peptide to peel off template.

tion for reaction with its activated amino acid. Persumably in this case the last position would be specific for a simple amino acid, i.e., having a free carboxyl group, which then detaches the completed peptide chain from the template when its amino group is acylated.

Although a covalent bond was shown in the illustrative example, other permutations are possible. The attached, e.g., tenth, residue of the peptide might be present as a tightly bound adenylate. As the next, i.e., eleventh, acyl adenylate arrives, its amino group reacts to form a peptide bond, and its adenylated carboxyl group is then tightly bound. Or the tightly bound groups could be at the amino end of the peptide, in which cases elongation of the peptide would occur by displacement by this amino group in the carbonyl of the incoming amino acid anhydride. The key features of the mechanism are, therefore, that the end of the completed portion of the peptide chain is tightly bound to the template; that this tight bond is released upon formation of the peptide bond with the subsequent amino acid; and that each peptide bond formed "induces" a new template area, which allows the formation of the next bond.

This chemical model is consistent with the template model. It explains how a zipper-type action could occur, since each link added to the chain establishes the conditions for the next link. It explains how the peptide chain can peel off the template as each link is completed. The peeling-off occurs because the formation of the peptide bond causes a dramatic change in chemical type. In the example shown, the covalent bond is broken. In the case of a strongly attracted amino

acyl-$X$ or $-\overset{+}{N}H_3$, either the $X$ is released or the positive charge is removed. Hence a template affinity strongly dependent on $X$ or the positive charge would be eliminated. As a result, the intramolecular attractions in both the template and the completed peptide would probably become more important than the template-peptide attraction. The peeling-off of the peptide chain and the return of the template to its original condition then allow the initiation of new chains before the completion of the old.

This model also explains other phenomena which are not a necessary concomitant of the zipper concept. For example, a zipper-type model does not provide any necessary reason for the absence of intermediates on failure to supply one amino acid. There would be no reason to keep the template from linking residues until a position requiring the deficient one was reached, in which case the partially completed peptide chain would accumulate. However, the concept that the growing end of the chain is always tightly bound to the template unless released by the subsequent amino acid means that a deficiency would leave the template "poisoned" by its own partially completed chain. Thus intermediates could only accumulate in amounts equal to the concentration of template, which is very small. Moreover, the chain could not start in the middle and proceed to the end, since the initiating site has not been "induced" into its proper orientation.

Finally, it should be stated that the author is well aware that this theory is not a complete answer to protein synthesis. It is, however, as far as he knows, compatible with the existing facts and leads to concrete predictions, e.g., under conditions where asymmetric labeling is observed, a particular amino acid should have progressively increasing specific activity on going down a chain. Also the combination of

data from the action of pure enzymes with data on timing in in vivo systems puts some chemical flesh on the mechanical skeleton of the template.

*Summary.*—The problem of protein synthesis is formidable because of a combination of three requirements, each one of which by itself is rather easily satisfied. Since the conditions are satisfied in each case by an enzyme-catalyzed reaction, the "template" in protein synthesis is presumed to be enzyme-like in character. Recently a new theory to explain enzyme specificity has been proposed which suggests that reaction between enzyme and substrate can occur only after a change in protein structure induced by the substrate itself. When this theory is applied to the problem of protein synthesis, it is seen that the existing data can be explained by a flexible template in which each completed peptide bond induces an alignment necessary to the formation of the next bond.

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# *CHEMICAL AERONOMY**

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*Introduction.*—The character of the earth's atmosphere is due mainly to the influence of the solar radiation on the gases held by the gravitational attraction of the earth. To understand this influence, we shall first briefly review the spectral distribution of the sun's radiation and its effect on the physical properties of the earth's atmosphere. Chemical aeronomy, or the study of the chemical reactions taking place in the upper atmosphere, will then be discussed, together with the role it plays in producing the night airglow. Finally, the technique of using chemical reactions to determine physical properties of the upper atmosphere, using rockets, will be introduced.

*Solar Radiation.*—The spectrum of the sun's radiation in the visible and infrared regions is approximately that of a black body at 5800° K.[1] In the ultraviolet portion of the spectrum, however, the solar continuum corresponds to a cooler black body. For example, in the spectral region from 1300 to 1750 A, it approximates a black body at 4500° K.[2] Discrete radiation from the sun is often more intense than the continuous spectrum. Hydrogen Lyman-alpha radiation at 1216 A, for example, is 10 to 100 times more intense than the continuum in this region.[3] The sun also radiates in the far-ultraviolet and X-ray regions. This energy may be approximated by a gray body at 700,000° K.[4]

*Physical Aeronomy.*—The physical structure of the atmosphere results from the combined effect of the earth's gravitational field and the direct and indirect absorption of radiation from the sun. The study of the physical properties of the atmosphere, such as pressure, temperature, density, winds, and electron densities, might be called *physical aeronomy*. The data on some of these physical properties are shown in Figure 1. At the left, the electron density in the ionosphere is plotted versus altitude.[5] This curve shows the continuous distribution of electrons into the D, E, and F regions. At the extreme right of Figure 1, the values of pressure in the atmosphere are given from rocket measurements.[6] The temperature curve at the right of center in Figure 1 is derived from a study of these rocket pressure measurements.[6] The nomenclature of the various regions of the atmosphere based on the temperature curve are given.[7] The troposphere is the lowest region of the atmosphere where the temperature decreases with increase in height. The isothermal region directly above the troposphere is the stratosphere. The mesosphere is the region between the top of the stratosphere and the major minimum of temperature at about 80 km. The region of increasing temperature above the mesosphere is called the thermosphere. The upper fringe of the atmosphere, where the atmospheric constituents no longer behave as a gas but as individual particles, is the exosphere.

*Chemical Aeronomy.*—The chemical structure of the atmosphere is also mainly the result of the action of solar radiation on the atmospheric gases. In the region

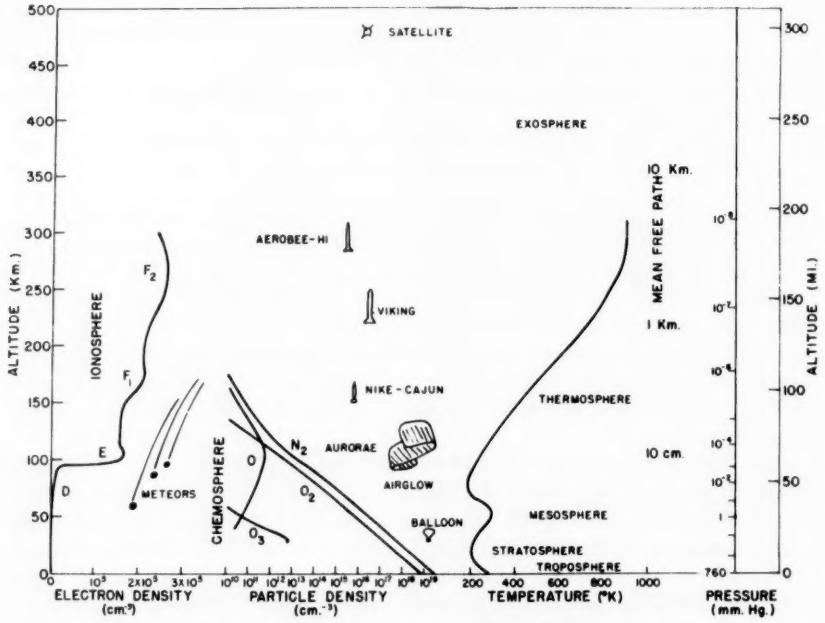


FIG. 1.—Properties of the upper atmosphere.

of the atmosphere near 30 km., ozone is formed through the absorption of solar ultraviolet radiation by molecular oxygen. The particle density of ozone is shown in Figure 1. The ozone concentration is in photochemical equilibrium between 35 and 70 km.[8]

The dissociation of molecular oxygen by solar ultraviolet radiation also produces an atomic oxygen region near 100 km. Above this level the atomic oxygen concentration is greater than the concentration of molecular oxygen, as shown in Figure 1.[6] Photoequilibrium is not established, however. Atmospheric mixing and diffusion cause molecular oxygen to be found at higher levels and atomic oxygen at lower levels than photochemical equilibrium would predict.[9]

In contrast to oxygen, nitrogen does not possess a strong dissociation continuum in a spectral region where the solar radiation is intense. Hence molecular nitrogen is found at much greater heights than molecular oxygen. The sources of atomic nitrogen are indirect and weak ones. In the ionosphere, molecular nitrogen is ionized by far-ultraviolet and X-radiation. Dissociative recombination of the ionized nitrogen produces atomic nitrogen in this region.[10] It has been shown that the nitrogen atoms will then diffuse down into the chemosphere.[11] Lyman-alpha radiation at 1216 A penetrates deeper into the atmosphere and may produce a low concentration of nitrogen atoms above 75 km. through a weak predissociation mechanism.[12]

These different atmosphere constituents—atomic oxygen, atomic nitrogen, molecular oxygen, molecular nitrogen, and ozone—react chemically with one another. This chemical activity has led to the designation of this region of the

atmosphere as the *chemosphere*[13] and of the study of these reactions as *chemical aeronomy*. The chemosphere may be designated as starting at the bottom of the ozone region and extending upward to the highest level where molecular oxygen is still present, as indicated in Figure 1.

The action of far-ultraviolet and X-radiation on the atomic oxygen, atomic nitrogen, molecular oxygen, and molecular nitrogen in the atmosphere is able to account for the density of electrons found in the E and F regions of the ionosphere. In order to explain the ionization found in the D region, however, it is necessary to assume that nitric oxide molecules are present in the chemosphere near 75 km. The nitric oxide is ionized by Lyman-alpha radiation, which penetrates to this level. It has been estimated that there must be a concentration of about $10^9$ molecules/cm$^3$ near the 75-km. level.[14]

It is important, then, to see what the sources of nitric oxide may be in this region of the atmosphere. It is possible to estimate the roles of the various aeronomic chemical reactions in the formation of nitric oxide in the chemosphere by using recent laboratory data on their reaction rates.[15] The most important chemical source of nitric oxide in the upper atmosphere is the reaction between atomic nitrogen and molecular oxygen,

$$N + O_2 \rightarrow NO + O, \tag{1}$$

which takes place near the top of the chemosphere. Rocket experiments have shown both atomic nitrogen and molecular oxygen to be present near 130–140 km.[16, 17] The three-body reaction between atomic nitrogen and atomic oxygen,

$$N + O + M \rightarrow NO + M, \tag{2}$$

may contribute to the production of nitric oxide at a lower level near 80 km.[18] Still lower in the chemosphere, atomic nitrogen may react with ozone to produce nitric oxide,

$$N + O_3 \rightarrow NO + O_2, \tag{3}$$

depending on how deeply atomic nitrogen penetrates into the ozone region.[19]

Once the nitric oxide is present in the chemosphere, it will react with atomic oxygen to form nitrogen dioxide:[20]

$$NO + O \rightarrow NO_2 + h\nu. \tag{4}$$

The nitrogen dioxide that is formed will also react with atomic oxygen, rejuvenating the nitric oxide,

$$NO_2 + O \rightarrow NO + O_2. \tag{5}$$

During the day, the nitrogen dioxide will also be dissociated by solar radiation of wave length less than 3700 A,

$$NO_2 + h\nu \rightarrow NO + O. \tag{6}$$

The reaction rates are such that there will always be a larger concentration of nitric oxide present than of nitrogen dioxide:[21]

$$n(NO) > n(NO_2).$$

The only chemical reactions which remove nitric oxide and nitrogen dioxide

from the upper atmosphere are (1) the photodissociation of nitric oxide by radiation less than 1900 A in wave length,

$$NO + h\nu \rightarrow N + O, \tag{7}$$

and (2) the reactions of nitric oxide and nitrogen dioxide with atomic nitrogen,

$$NO + N \rightarrow N_2 + O, \tag{8}$$

$$NO_2 + N \rightarrow N_2 + O + O. \tag{9}$$

Reactions (8) and (9) are the only ones that return the nitrogen-oxygen compounds to stable forms. The reaction rates and particle concentrations are such that reaction (8) is far more important than reaction (9).[15, 21]

An examination of these aeronomic chemical reactions indicates that reaction (1) between atomic nitrogen and molecular oxygen,

$$N + O_2 \rightarrow NO + O, \tag{1}$$

and reaction (8) between atomic nitrogen and nitric oxide govern the concentration of nitric oxide in the chemosphere.[21] When the rates of formation and destruction of nitric oxide by these reactions are equated, the equilibrium nitric oxide concentration is independent of the atomic nitrogen concentration but is proportional to the concentration of molecular oxygen:

$$\frac{dn(NO)}{dt} = K_1 n(N) n(O_2) = K_7 n(N) n(NO),$$

$$n(NO) = \frac{K_1}{K_7} n(O_2), \qquad \text{where } K_1 < K_7.$$

This means that the greatest concentration of nitric oxide will be found at the lowest point in the chemosphere where atomic nitrogen is present. Since chemical equilibrium may not be established, diffusion may play a role in bringing nitric oxide to still lower levels. Any nitric oxide diffusing upward would be destroyed by the increased atomic nitrogen concentration, while the nitric oxide diffusing downward appears to be immune from chemical reactions that would convert it back into molecular nitrogen and molecular oxygen.

*The Night Airglow.*—In many of the chemical reactions taking place in the atmosphere, the energy released appears in the form of luminous emission from the atoms or molecules produced by the reaction. The atomic lines and molecular bands in the night airglow serve as a source of information as to what chemical reactions are taking place in the high atmosphere and what the physical conditions are at the level of emission.

In the visible region of the spectrum, the main spectral features are the atomic lines of oxygen at 5577 A and 6300 A and the D lines of sodium at 5893 A. The height of emission of the 5577 A oxygen line has been measured by a photometer flown in a rocket. The maximum intensity of this emission was found to be near 100 km., which clearly supports Chapman's early proposal that the origin of the 5577 A line in the nightglow is due to the three-body recombination of atomic oxygen.[22] The emission altitude of the sodium D lines has also been measured by a rocket-flown photometer. Its maximum intensity near 85 km. indicates that a chemical mechanism must be responsible for this excitation.[22]

The Herzberg oxygen bands are the principal feature of the night airglow in the ultraviolet portion of the spectrum, and the (0, 1) atmospheric oxygen band is a prominent feature in the infrared.[23] The most likely chemical reaction exciting both these systems of bands in the upper atmosphere is the three-body recombination of atomic oxygen,

$$O + O + M \rightarrow O_2^* + M. \quad (10)$$

These bands have been produced by this reaction in the laboratory. In Figure 2,

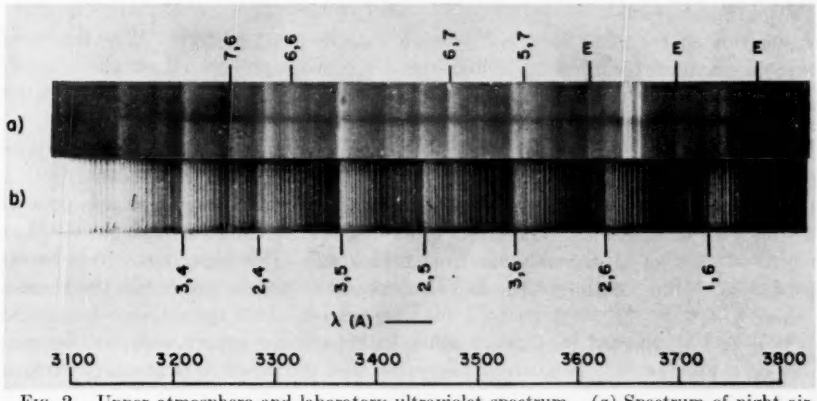


FIG. 2.—Upper atmosphere and laboratory ultraviolet spectrum. (*a*) Spectrum of night airglow, showing Herzberg oxygen bands and unknown E bands (see n.24). (*b*) Spectrum of laboratory oxygen afterglow, also showing Herzberg bands and unknown E Bands.

a spectrogram of the ultraviolet night airglow[24] is compared with a spectrogram obtained from a laboratory afterglow produced by the recombination of oxygen atoms. The Herzberg oxygen bands appear in both spectra. The differences in the intensity distribution of the vibrational bands and rotational lines are due to the differences in temperature and pressure between the two sources.[21] The upper spectrogram also shows several still unidentified features in the night airglow spectra. These features have now been produced in the laboratory also and appear in the lower spectrogram. Thus it is expected that these last unknown emissions of the night airglow will soon be identified. Both the concentration of atomic oxygen in the chemosphere and the rotational temperature of the Herzberg and atmospheric bands in the airglow predict a level of emission near 80 km.

The Meinel vibration-rotation bands of the hydride radical are the principal features of the night airglow in the infrared.[23] The excitation of these bands has been attributed to the reaction between atomic hydrogen and ozone,

$$H + O_3 \rightarrow OH^* + O_2. \quad (11)$$

This reaction has been found to produce these bands in the laboratory.[25] The temperature of the OH bands and the atmospheric distribution of the reactants is compatible with a level of emission near 70 km. However, there has been another OH excitation mechanism proposed, which predicts a high level of emission.[26] The correct explanation could be determined if the level of emission of the

OH bands were measured from a rocket. This might be best accomplished by using an infrared detector that is sensitive to 2.7 $\mu$ radiation where the emission from the OH radical should be the most intense.

*Rocket-Seeding Experiments.*—Recently, artificial airglows have been produced in rocket experiments. This technique permits the detection of some of the atomic constituents of the upper atmosphere by rocket seeding. In its simplest form, this method consists of first finding a luminous chemical reaction which has as one of its reactants the atmospheric constituent that is to be detected. The radiation given off by this reaction should consist preferably of discrete spectral lines or bands. The chemical reaction is set off at the level of the upper atmosphere under investigation by releasing the other reactant from a rocket at night. The luminous emission is then recorded by a high-speed spectrograph located on the ground. The fact that the reaction takes place and does emit light is evidence that the atmospheric reactant is present in that region of the atmosphere.

The Geophysics Research Directorate has detected atomic nitrogen in the upper atmosphere by a rocket-seeding experiment.[16] Ethylene gas was released from a rocket, and the resulting luminescence indicated that atomic nitrogen was present at the level of emission. The GRD group has also produced a luminous cloud at night by ejecting nitric oxide gas from a rocket.[27] This experiment may be regarded as an illumination of the fact that atomic oxygen is present in the chemosphere.

It is to be expected that many other rocket-seeding experiments will be performed in the future. It has been suggested that the presence of atomic hydrogen in the upper atmosphere may be verified by releasing ozone from a rocket into the chemosphere.[28] The resulting reaction between atomic hydrogen and ozone should produce the vibration-rotation bands of OH. This emission could be detected in the infrared region of the spectrum.

Using the technique of creating an artificial airglow by rocket seeding, it should be possible to measure the temperature of the atmosphere at different heights. For this purpose, it is necessary that a product of the reaction be an excited molecule that is sufficiently metastable to come into rotational equilibrium with the ambient air. The atmospheric band of oxygen has this property. The band is the result of a magnetic dipole transition and has a lifetime of about 7 seconds.[29] It has recently been shown in the laboratory that it is excited in the reaction between nitrogen dioxide and atomic oxygen.[30] Thus the atmospheric oxygen band could be excited in the upper atmosphere by releasing nitrogen dioxide gas from a rocket into the chemosphere. This technique is applicable in the region of the atmosphere between 80 and 140 km. Figure 3 shows two spectrograms of the atmospheric oxygen band taken in laboratory oxygen afterglows. In the upper spectrogram the afterglow was cooled to 200° K., and in the lower spectrogram the afterglow was heated to about 650° K. This is the temperature range that is of interest in the region of the thermosphere where this method is applicable. The shift in the intensity of the rotational lines is readily apparent. The atomic hydrogen–ozone reaction could also be used to measure temperatures in the atomic hydrogen region.

In conclusion it should be pointed out that the technique of studying the properties of an inaccessible region by exciting the spectra of its constituents through a

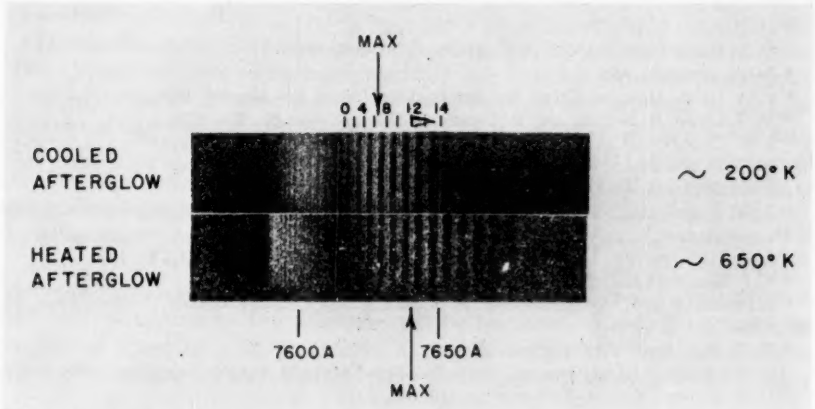


FIG. 3.—(0,0) atmospheric oxygen band. *Upper spectrogram*, oxygen afterglow cooled with dry ice; *lower spectrogram*, oxygen afterglow heated by external nichrome wire.

chemical reaction is of general applicability. For example, the first experiment to determine whether or not there is oxygen in the atmosphere of Mars might be performed by releasing hydrogen gas into the Martian atmosphere from a rocket and spectroscopically examining any resulting emission for bands of OH. Thus it appears that chemical aeronomy will be useful and productive for a long time into the future.

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# *INDUCTION OF SPECIFIC MUTATIONS WITH 5-BROMOURACIL**

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## INTRODUCTION

The hereditary characteristics of an organism occasionally undergo abrupt changes (mutations), and genetic techniques have traced these to alterations at definite locations in the genetic structure. Recently, the fineness of this genetic mapping has been extended to the level where the finite molecular units (nucleotides) of the hereditary material limit further subdivision. At this level, local details of the hereditary material should exert their influence; the frequency of mutation at a particular point should depend upon the local molecular configuration. It is therefore feasible to try to correlate genetic observations with precise molecular models, such as the one proposed by Watson and Crick[1] for the structure of DNA.

In a fine-structure study of spontaneous mutations in phage T4, the mutability at different points in the genetic structure was, in fact, found to be strikingly varied.[2] To relate mutability to actual chemical structure, it would seem promising to employ mutagenic agents of specific types, to act selectively on particular configurations. Since the initial discovery by Muller[3] and Stadler[4] on induction of mutations with X-rays and the discovery of chemical mutagenesis by Auerbach and Robson[5] and by Oehlkers,[6] many physical agents and chemical substances have been found to be mutagenic in many organisms. Some mutagens act selectively; in particular the induced reversion from biochemically dependent to independent strains has been shown to depend upon the mutant and the mutagen used. (For chemical mutagens in bacteria see Demerec.[7]) A recent comprehensive review of this subject has been published by Westergaard.[8] Mutagens in some cases produce gross chromosomal aberrations; in others the alterations are so small as to

be beyond the limited resolving power of genetic techniques for the organism used.

The absence of this limitation makes phage a suitable organism for our purposes. There have been reports of induction of mutations in phage by ultraviolet light,[9,10] nitrogen mustard,[11] streptomycin,[12] and proflavine.[13] A very provocative discovery is that analogues of the normal bases may be built into DNA in place of the usual ones and also raise the mutation rate. In particular, one such analogue, 5-bromouracil, has been proven by Dunn and Smith[14] to be incorporated into the DNA of phage (in place of thymine), and Litman and Pardee[15] have shown that it greatly increases the frequency with which phage mutants of various types arise.

In the present work, this possibility of directly affecting the DNA structure is combined with a genetic analysis of high resolving power, to make a fine-structure study of mutagenesis. Our attention will be restricted to the *r*II region of the genome of phage T4. The mutational alterations arising by 5-bromouracil induction are compared to, and shown to differ from, those which occur spontaneously.

## METHODS AND MATERIALS

Strains: *phage* T4B; *bacterium* B (*E. coli* B) for the isolation of mutants and as plating bacterium for the determination of phage titers; S (*E. coli* K12S) for the preparation of phage stocks; K (*E. coli* K12S lysogenic for prophage lambda) as the selective strain for genetic tests.

Media: *broth* 1 per cent bacto-tryptone (Difco) plus 0.5 per cent NaCl; *glucose-salts* medium;[16] *sulfanilamide medium* same as used by Litman and Pardee,[15] except for higher sulfanilamide concentration (2 mg/ml) and addition of 1 $\mu$g/ml calcium pantothenate, 1 $\mu$g/ml pyridoxine, 1 $\mu$g/ml thiamine, 1 $\mu$g/ml uracil, and 20 $\mu$g/ml L-tryptophane (tryptophane required for adsorption of phage T4B to B in synthetic medium). Plates contain *broth* plus 1.3 per cent agar (Difco) with a top layer of broth plus 0.7 per cent agar.

A sample of *5-bromouracil* purified by ion-exchange column, was kindly supplied by Dr. Rose Litman.

*Isolation of the Mutants.*—Spontaneous mutants: Details on the isolation of spontaneously arising *r* mutants of phage T4, their properties, and the methods used in mapping them genetically are given in earlier publications.[2, 17] In brief, a stock of standard ("wild") type phage T4 (derived from a single T4 particle) is plated on B. Each phage particle produces a plaque containing around $10^7$ progeny. The progeny include occasional *r* mutants, which can be found by picking the plaque and replating its contents. In order to assure that each mutant arises by an independent mutational event, no more than one *r* mutant is isolated from any one plaque of the standard type. Each *r* mutant is replated (to free it from any contaminating particles of standard type), an isolated *r*-type plaque is picked, and a stock of the mutant grown on bacteriums in broth.

Induced mutants: These were isolated from the yield of bacteria infected and allowed to burst in the presence of sulfanilamide and 5-bromouracil. A culture of B was prepared by inoculation of 0.4 ml. of overnight culture (grown in glucose-salts medium) into 20 ml. of sulfanilamide medium and aeration for 3.5 hours to reach a cell concentration of $7 \times 10^8$ per ml. At this time, 1 mg. of 5-bromouracil and $4 \times 10^4$ particles of T4 standard-type phage[18] were introduced simultaneously. Drops

of the mixture were rapidly distributed—one drop into each of 200 tubes. After incubation at 37° C. (for 30 minutes) to allow the infected cells to burst, the content of each tube was plated on B. As is typical in this sulfanilamide medium, the average yield per cell was very small, of the order of one viable progeny particle per infected cell. Each plate contained (after incubation) from 30 to 100 plaques, in most cases including one or more *r*-type plaques. The over-all proportion of *r*-type particles among the progeny was about 2 per cent. To assure the independent origin of each mutant, no more than one *r* plaque was picked from a plate. Each such mutant was purified by replating, and a stock prepared on S in broth.

*Genetic Mapping of the Mutants.*—Different *r* mutants of T4, although producing similar plaques on B, fall into groups distinguished by their behavior on a second host, K. Those mutants with which we are here concerned, of the *r*II group, do not produce plaques on K. This property is the key to the high resolution with which they can be mapped genetically. When two *r*II mutants are crossed, the appearance of any standard-type recombinants among the progeny is readily detected by plating on K. If standard-type progeny *are* produced in a cross (above the background rate due to spontaneous reversion of the mutants), it is concluded that the two mutants contain alterations at different locations in their genetic structures.

Our objective is to compare these locations for spontaneously arising and for 5-bromouracil-induced mutants. The task of crossing a large number of mutants, two by two, to see which pairs yield recombinants is enormous. However, this process can be shortened by making use of a set of mutants having large alterations, as shown in Figure 1. Each new isolated *r*II mutant is crossed with each mutant of this set (by means of simple spot tests). By noting with which mutants of the set it does or does not produce standard-type recombinants, the mutation can be assigned to a particular *segment* of the map. Thereafter, only mutants belonging to the *same* segment need be crossed with each other. Thus the number of crosses required for analyzing a batch of mutants is greatly reduced.

The genetic procedure has therefore been to (1) isolate many independently arising *r*-type mutants; (2) choose those of the *r*II group; (3) test each *r*II mutant against the mutants of Figure 1, thereby locating its mutational alteration in a particular segment of the map; and (4) cross the mutants belonging to the same segment with each other to determine which mutations share common locations. For the present purposes, no attempt was made to determine the *order* of these locations *within* a map segment.

*Reversion Rates of Mutants.*—The different *r*II are characterized not only by the positions of their mutational alterations in the map but also by differences in their frequency of reversion to particles that resemble the standard type in their plating behavior on K and B. These revertants arise spontaneously during the growth of a given *r*-type phage, and their typical frequency, in a stock grown up from an inoculum of 100 *r* phages, is called the "reversion index."

## RESULTS

*Over-all Mutation Frequencies.*—The ratio of the induced to the spontaneous rate of mutation cannot be given accurately, since different procedures of isolation were used. A rough estimate may be made as follows: In a broth lysate of T4B grown (on S) from an inoculum of a few particles up to a population of $10^{10}$, the proportion

of spontaneously appearing *r* particles is typically $2 \times 10^{-4}$. Under the conditions used for induction, on the other hand, *r* mutants appeared in a proportion of around 2 per cent in the progeny from a single growth cycle. To obtain the probability of mutation *per phage replication*, these values must in each case be adjusted to account for the accumulation of mutants during the growth of the population. This effect, which is roughly proportional to the logarithm of the ratio of final to initial phage titer, should be several fold (perhaps four times) larger for a lysate than for one cycle of growth. Therefore, the rate of induced over-all *r* mutations can be estimated as several hundred times the spontaneous rate.

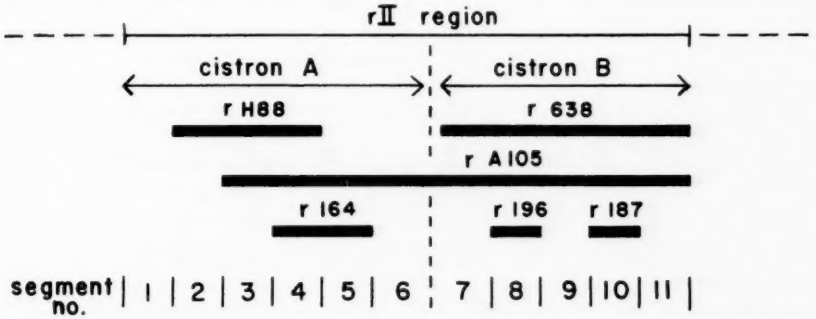


FIG. 1.—Genetic map of the *r*II region of phage T4, showing the division of the map into segments by a set of *r*II mutants having large alterations. Each of the two "cistrons" is a functional unit defined by the cis-trans test.

The relative positions of the alterations have been established by their overlapping relationships with a much larger set of mutants. To locate the defect of a new mutant, it is crossed with each member of the set, and standard-type recombinants are tested for. From the results of these crosses the defect can be assigned to one of the segments. For example, if a mutant gives standard-type recombinants with all except *r* H88, *r* A105, and *r* 164, its defect must be located in segment 4. One which gives recombinants with all must have its defect in segment 1. Segments 7, 9, and 11 are not distinguished by this set of mutants.

*Genetic Analysis of the Mutants.*—Spontaneous mutants: A series of 238 spontaneously arising mutants was isolated, numbered *r* 101 through *r* 338, and analyzed as described above. Of the mutants, 132 belonged to the *r*II group. Of these, 6 were not readily mappable because of high reversion rates or leakiness (ability to grow feebly on K). The results of mapping the remaining 126 mutants are shown in Figure 2.

The relative positions of the sites indicated *within* a map segment have no significance. Any two mutants that yield standard type progeny when crossed are sketched as horizontally separated, while those groups for which recombination could not be detected (at a level of about 0.01 per cent recombinant progeny or more) are assigned to the same or adjacent horizontal sites. Some mutants fail to give recombinants with (any of two or more) other mutants known to give recombinants with each other; the alterations of such mutants are represented as horizontal bars. A striking feature of the map is the existence of certain "hot spots," where mutations recur with high probability.

Induced mutants: A series of 170 *r* mutants, arising under the action of 5-bromouracil in sulfanilamide medium, was numbered N 1 through N 170. Of these, 89 were of the *r*II type. Twelve of the latter were not readily mappable because

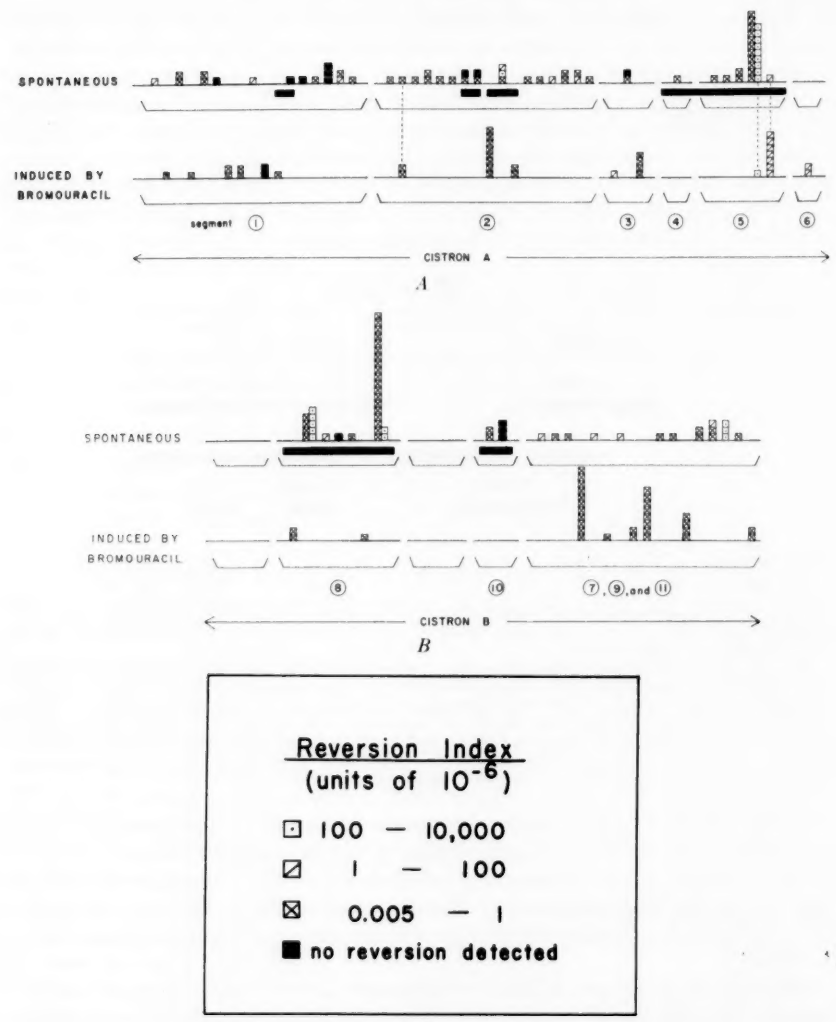


FIG. 2.—Genetic maps showing the locations of spontaneous and 5-bromouracil-induced mutational alterations in the *r*II region of phage T4 divided into cistron A and B. Each mutant is represented by a box, placed in the proper segment of the map and shaded to indicate its reversion index. The relative order (but not the lengths) of segments 1, 2, 3, 4, 5, 6, 8, and 10 have been established. Segments 7, 9, and 11, are lumped together and considered as one segment. The order and position of different mutations within one segment have not been determined.

they were leaky. The results of mapping the remaining 67 mutants are shown in Figure 2. This set of mutants reveals "5-bromouracil hot spots" that are located at different positions from the spontaneous ones.

It must be emphasized that the vertical scales in Figure 2 are very different for the two sets of mutants. The over-all induced mutation rate was several hundred-

fold higher, and only half as many induced mutants are mapped; therefore, the occurrence of an induced mutant once represented in the induced set corresponds to roughly $10^3$ times higher mutability than the occurrence of a spontaneous mutant once represented in the spontaneous set. Hence, for those "hot spots" in the induced set for which no spontaneous mutation has been observed, the mutability with bromouracil is at least $10^4$ times larger than is the spontaneous mutability.

*Reverse Mutations.*—Among the spontaneous *r*II mutants, not only are forward mutation frequencies different for different sites, but the reversion indexes as well cover an enormous range. Among the 132 spontaneous *r*II mutants, reversion indexes range as high as $5 \times 10^{-2}$ down to less than $10^{-8}$. For 19 of them, reversion has not been detected.

The induced mutants, on the other hand, are more homogeneous with respect to reversion index. There is only one mutant of very high reversion index (N 76) and two non-reverting mutants (N 101, N 32). Most of the remainder have reversion indexes of the order of $10^{-8}$. Notably rare are mutants containing larger aberrations (i.e., ones that fail to give standard-type recombinants with two or more other mutants that do themselves show recombination).

Some preliminary experiments have been performed on the induction of reversion of various mutants with 5-bromouracil. For some mutants, in particular, chosen from some of the "5-bromouracil hot spots," increases over the spontaneous rate of reversion by factors as high as $10^4$ have been observed. Some spontaneous mutants show no or only much smaller effects. These experiments are still in progress.

## DISCUSSION

Clearly, the mutagenic effect of 5-bromouracil is not merely a general enhancement of spontaneously occurring mutations. Rather, it is a *specific* effect. In denoting the action of a mutagen as specific, one might be comparing the effects on different phenotypic characteristics in the whole organism, different cistrons, or different locations within a single cistron. Were it not for the high resolution of our genetic techniques, it might have been erroneously concluded that 5-bromouracil acts aspecifically, since, among all the induced mutants of the *r* phenotype, the proportion of *r*II mutants and even the ratio of A cistron to B cistron mutants are quite comparable to the spontaneous values. It is only at finer resolution that the different effects are revealed, and it is seen that mutations are, for the most part, induced at specific places in the genetic structure.

Since the proportion of large aberrations and non-reverting mutants is notably smaller among the 5-bromouracil-induced mutations, the induced changes in the genetic structure appear to involve small molecular substitutions rather than large changes of the genome. Further, the more homogeneous properties of the induced mutants with respect to reversion rates indicate that a certain class of molecular transitions may be involved here.

Under the conditions used, the *total* frequency of occurrence of all *r*II mutants in 5-bromouracil was raised several hundred fold above the spontaneous rate. The increase in rate at specific locations in the genetic structure was much larger. For example, 11 occurrences of mutation at one site were observed out of 67 induced *r*II mutants, while none occurred at that site among twice as many spontaneous *r*II

mutants. Thus the probability of mutation at that point, per replication of the phage, was raised by a factor greater than twice eleven times several hundred, or of the order of $10^4$.

Conversely, note the site at which there were 19 occurrences among 132 spontaneous *r*II mutants, while among the 67 induced mutants none were observed. Therefore, 5-bromouracil had little, if any, positive mutagenic effect at that point. The disappearance of the spontaneous "hot spot" does not, of course, mean that spontaneous mutations at that point were suppressed but simply that they were not increased in proportion to other mutations.

Only three sites (indicated by dotted lines in the figure) are represented in both sets of mutants. In each of those cases, there was only a single occurrence in one of the sets, so that the ratio of induced to spontaneous frequencies cannot be reliably computed. The mutation rate seems to have been truly increased by 5-bromouracil in 2 of the cases (2 and 7 occurrences in the induced set) because one occurrence among the induced series represents a thousandfold greater probability of mutation. In the third case, there is, in the induced set, a single occurrence of a highly revertable mutant that appearaed nine times in the spontaneous set. This single event probably belongs to the small background of spontaneous mutations among the induced ones (*ca* 1 or 2 in 100).

The methods used in the isolation of the two sets of mutants were quite different, and one cannot be sure whether factors other than the addition of 5-bromouracil could have affected the results. Although sulfanilamide alone does not appreciably increase the total mutation rate, the possibility that the distribution of mutations might change has not been excluded. Sulfanilamide, as an analogue of *p*-amino benzoic acid, inhibits the formation of folic acid, thereby inhibiting nearly all the methylation and hydroxymethylation steps (Cohen and Barner).[19] In our sulfanilamide medium, nearly all the major chemicals containing the methyl or hydroxymethyl group are added except the deoxyribonucleotides of thymine and 5-hydroxymethylcytosine, which thus are expected to be deficient. The deficiency in thymine facilitates the incorporation of 5-bromouracil into DNA. Whether the deficiency in 5-hydroxymethylcytosine enhances the probability of the false incorporation of 5-bromouracil into a hydroxymethylcytosine site of the phage DNA remains to be seen.

One is not in a position, from these experiments alone, to reach a clear conclusion as to the molecular mechanism of mutagenesis; thus, at this stage, it cannot be decided whether or not the tautomeric shift of the DNA bases from the *keto-* to the *enol-* form is mainly responsible for the production of point mutations, as suggested by Watson and Crick.[1] If it is assumed that only two kinds of nucleotide pairs are present in DNA (i.e., adenine—thymine, and guanine—5-hydroxymethylcytosine), the existance of hot spots of spontaneous mutation and the appearance of different hot spots with 5-bromouracil would suggest that not every nucleotide pair of a given type mutates with the same probability. Rather, the mutability of a nucleotide pair would have to depend upon its position.

In any case, the striking results of this preliminary investigation indicate that it would be fruitful to pursue this line of investigation, using a range of mutagenic substances in systems where the chemical events are under proper control.

SUMMARY

A set of spontaneously arising *r*II mutants of phage T4 is compared with a set of mutants induced by the action of 5-bromouracil. When analyzed by genetic mapping techniques of high resolution, the two sets of mutants are found to be quite different. The mutagen does not merely enhance the over-all mutation rate but acts at specific locations in the hereditary structure.

The induced mutants are mostly of the nature of small, revertible alterations rather than gross defects. The reversion rates of induced mutants are less varied than those of spontaneous mutants, indicating that a certain class of molecular transition is involved.

These preliminary results encourage the hope that this sort of genetic analysis can lead toward an understanding of the mechanism of mutation and the identification of the specific chemical configurations composing the genetic structure.

*Note:* The occurrence at a given location of one mutation in the induced set corresponds to *roughly $10^3$ times higher mutability* of this spot in 5-bromouracil than is indicated by the occurrence of one mutation in the spontaneous set.

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# *A THEORY OF OPTIMUM CONDITIONS FOR PHASE TRANSITIONS**

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If one considers the transport of material between two phases, it is apparent that the activated state must, in effect, represent a bridge between them. It is further obvious that, though a very large number of possible reaction paths might be imagined, those most favorable energetically will virtually account for the entire transfer. At the present time it is premature to attempt a detailed description of the mechanism by which an atom or molecule may leave the surface of one phase and go into another phase. There is little doubt, however, that screw dislocations and the imperfections at the interface between solid phases facilitate such transfer.

It is possible, nevertheless, without a detailed knowledge of the mechanisms, to formulate a useful description of the processes in terms of absolute rate theory and, in fact, to use this to investigate, in so far as data are available, the nature of the bridges between phases.

Consider the transition between phases A and C. One assumes that this transition occurs by way of bridges which exist at the juxtaposition of the phases. Further assume that an equilibrium exists between active bridges—at which the transition can take place—and deactivated bridges. One assumes that this equilibrium is established too rapidly to be observed. Now the total number of bridges, $B_t$, is a constant given by

$$B_t = B_a + B_d.$$

Further, one may write the equilibrium constant between the active and the deactivated bridge thus:

$$K' = \frac{B_d}{B_a}.$$

From which one may obtain

$$B_a = \frac{B_t}{1 + K'}. \tag{1}$$

This argument has been applied to the equilibrium between active and denatured enzyme systems in the study of luminescence and other biological processes.[1] In fact, this equilibrium explains the temperature optima which are almost universally observed in biological reactions. Such optima arise because, although a change of temperature normally speeds up a reaction, as Arrhenius emphasized, this speeding-up ceases when the rising temperature deactivates enzymes and so slows reactions more than the normal temperature gain. The replication of crystals is like biological replication of organisms in some other respects, but this presence of optimum conditions with parallel explanations is, perhaps, the most extraordinary resemblance.

The velocity of the phase transition is then given by

$$v = B_a\lambda(k_f - k_b) = B_a\lambda k_f\left(1 - \frac{1}{K}\right), \tag{2}$$

where $\lambda$ is the displacement of the interface for each molecular unit of reaction and $k_f$ is the specific rate constant of the reaction $C \rightarrow A$, while $k_b$ is the specific rate for $A \rightarrow C$. Thus, for a single type of bridge, we may write, from equations (1) and (2),

$$v = \frac{B_t\lambda}{1 + K'} k_f \left(1 - \frac{1}{K}\right). \tag{3}$$

Where a number of types of bridges are possible, the total velocity, $v_t$, will be given by

$$v_t = \sum_j \frac{B_{tj}\lambda_j}{1 + K_j'} k_{fj} \left(1 - \frac{1}{K_j}\right). \tag{4}$$

The effect of temperature, pressure, and other variables on the rate constant may be obtained from the absolute reaction-rate theory,

$$k' = \frac{\mathfrak{K}kT}{h} \exp\left(\frac{-\Delta F^{\neq}}{RT}\right). \tag{5}$$

For the pressure effect, one writes

$$k_p = k_0 \exp\left(\frac{-P\Delta V^{\neq}}{RT}\right). \tag{6}$$

In this expression $k_p$ is the absolute rate constant at pressure $P$, while $k_0$ is that at zero pressure. The term $\Delta V^{\neq}$ is the difference between the molal volumes of the activated and the initial state. The pressure effect on the equilibrium constant may be written thus:

$$K_p' = K_0' \exp\left(\frac{-P\Delta V'}{RT}\right) \tag{7}$$

and

$$K_p = K_0 \exp\left(\frac{-P\Delta V}{RT}\right). \tag{8}$$

In these expressions $\Delta V'$ is the molal volume of the deactivated bridge less that of the active bridge, and $\Delta V$ is the molal volume of the final state less that of the initial state. Equations (6), (7), and (8) are introduced into equation (4) if one desires to determine the effect of external pressure on the total velocity of the phase transition. Professor P. W. Bridgman has examined the effect of pressure on the velocity of some phase transitions of solids.[2]

This study disclosed two types of behavior. The simplest is that exhibited by ammonium thiocyanate (Fig. 1). In this case equation (4) may be used in the simplified form,

$$v = B_t \lambda k_{0j} \exp\left(\frac{-P\Delta V^{\neq}}{RT}\right)\left[1 - K_0^{-1} \exp\left(\frac{P\Delta V}{RT}\right)\right]. \quad (9)$$

If one employs Professor Bridgman's data, $\Delta V = -3.23$ cc/mole, and the values of $\Delta H$ and $\Delta S$ for the transition to calculate $K$, one obtains $\Delta V^{\neq} \doteq -17$ cc/mole. This gives a quite satisfactory fit between the theoretical curve calculated from equation (9) and the experimental data.

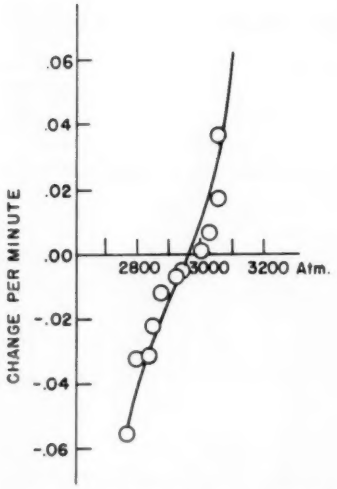


FIG. 1.—A plot of the fraction of total transition per minute versus pressure for ammonium thiocyanate at 0°C. The experimental points were reported by Bridgman. The curve was calculated using equation (9).

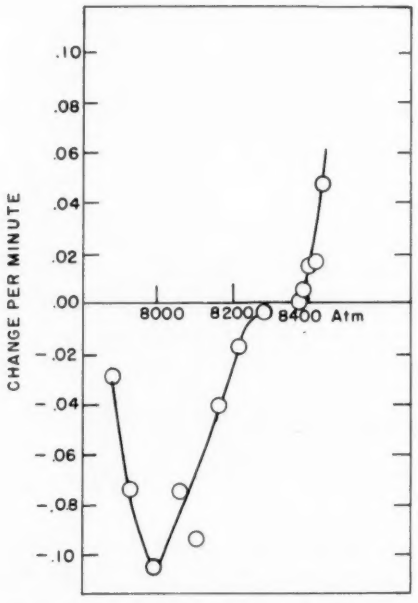


FIG. 2.—A plot of the fraction of total transition per minute versus pressure for silver nitrate at 75°C. This transition curve shows both a velocity maximum (minimum in the curve) and a region of indifference.

The case of silver nitrate (Fig. 2) is more complex. It will be noted that the velocity of transition as a function of pressure passes through a maximum and that there is a region of indifference (i.e., a region at velocity zero in which a change in pressure produces no apparent change in velocity). In this case we observe that the extremum condition from equation (4) is, for each term in the sum,

$$\frac{u'}{1+u'}\Delta V' - \Delta V^{\neq} - \frac{u}{1-u}\Delta V = 0, \quad (10)$$

in which

$$K_0' \exp\left(\frac{-P\Delta V'}{RT}\right) \equiv u'$$

and

$$K_0 \exp\left(\frac{-P\Delta V}{RT}\right) \equiv u.$$

Because the necessary temperature-dependent data are not available, it is not possible to evaluate the terms in equation (10). It is, however, apparent that the theory as outlined above predicts just such a maximum as was experimentally observed and correctly attributed by Bridgman to nucleation but which are here characterized as catalysts or bridges. The region of indifference would seem to arise from a second term in the sum of equation (4). The rate and equilibrium constants in the term are about the same as those in the first term, but the pressure region of stability of the new bridges is different. The second type of bridge is stable at elevated pressures, at which the first type have been reduced to a negligible concentration. Further, the pressure effect on the term $(1 + 1/K)$ has changed the sign of the velocity, and the reaction now proceeds in the opposite direction.

It would seem that a large number of phase transitions can be treated by the method outlined above. An accompanying paper uses the theory to explain an observed optimum of crystal growth of supercooled water on silver iodide crystals.

* This work was assisted by a grant from the National Science Foundation.

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## *THE VISUAL SYSTEM OF THE HONEYBEE**

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The insects, though a major group of animals possessing prominent eyes, have heretofore resisted attempts to learn something of the chemistry of their visual systems. They present unusual difficulties in this regard. Attempts by Wald to extract vitamin A from the heads of *Drosophila*, the grasshopper *Melanoplus*, and the dragonfly *Sympetrum* failed to yield a clear test for this substance, though relatively large amounts of tissue (6–14 gm.) were employed (unpublished experiments cited by Wald and Burg[1]). Similarly, Goodwin and Srisukh[2] have reported the absence of vitamin A in acetone extracts of whole *Locusta* and *Schistocerca*. Wolken[3] and Wolken *et al.*[4] have recently reported an unsuccessful attempt to extract a visual pigment from the heads of *Drosophila*.[5]

Recent experiments on the heads of the honeybee, *Apis mellifera*, however, have revealed the presence of retinene$_1$. Bodies and heads of bees were ground separately with anhydrous sodium sulfate or in some experiments were lyophilized, to dry the tissues. They were extracted first with petroleum ether and then with acetone, which is known to dissociate carotenoids from proteins, and retinene from all known visual pigments. The acetone extracts were evaporated to dryness under reduced pressure and taken up in petroleum ether. Four types of petroleum ether solution were prepared: (A) petroleum ether extract of bodies, (B) acetone

extract of bodies, (C) petroleum ether extract of heads, and (D) acetone extract of heads. These were chromatographed on columns of aluminum oxide (Merck Reagent, "Suitable for Chromatographic Adsorption") "weakened" by the addition of 5 per cent water. Yellow adsorption bands formed, which were eluted with 4, 10, and, finally, 40 per cent (*v/v*) acetone in petroleum ether. The various fractions were evaporated under reduced pressure, and each was taken up in 0.3 ml. chloroform. For the antimony chloride reaction, 0.25 ml. of the test solution was placed in an absorption cell in a Cary recording spectrophotometer. To this was added 1 drop of acetic anhydride and 0.5 ml. of a saturated solution of $SbCl_3$ in chloroform. The spectrum was recorded immediately.

The acetone extract of the heads (extract D) yielded the bright-blue color and absorption peak at 664 m$\mu$, which identifies retinene$_1$ (Fig. 1). The retinene was

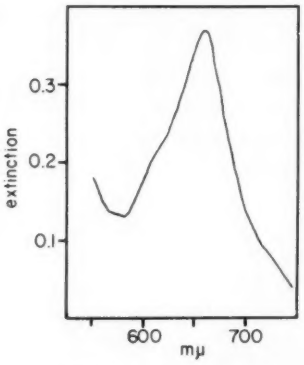


FIG. 1.—Antimony chloride test with a partly purified acetone extract of bee heads. The peak of absorption at about 660 m$\mu$ (which moves later to 666 m$\mu$) is specific for retinene$_1$. (This spectrum and those of Fig. 2 were traced from records drawn by the Cary recording spectrophotometer.)

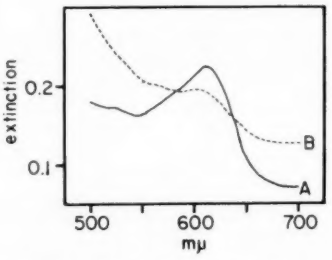


FIG. 2.—(*A*) Antimony chloride reaction of vitamin A, prepared by reducing with potassium borohydride the retinene extracted from bee heads. (*B*) The same, approximately 2 minutes later. Note the characteristic fading of the 618 m$\mu$ peak, evident despite the over-all rise in the base line.

found principally in the 10 per cent eluate. Since retinene has been found previously only in eye tissues, it is likely that in these experiments its source is the compound eyes and ocelli. No retinene was detected in the petroleum ether extract of heads (extract C, maximum of 14 gm. tissue extracted); apparently it is present only in bound form, presumably linked to protein, from which it is freed by acetone. A maximum of 0.22 $\mu$g. of retinene was obtained per gram fresh weight of heads. This corresponds to slightly more than $3 \times 10^{-6}$ micromoles per eye, which, per area of retina, is roughly 5 per cent the amount of visual pigment found in cattle. As expected, no retinene could be extracted from the bodies with either petroleum ether or acetone (extracts A and B, maximum of 45 gm. of tissue extracted).

The retinene was further identified by reducing its terminal aldehyde group with potassium borohydride, forming vitamin A.[6] On mixing with antimony chloride,

this yielded the characteristic blue product with an absorption peak at 618 mμ (Fig. 2).

Other carotenoids are present in these extracts, which will be described more fully later.

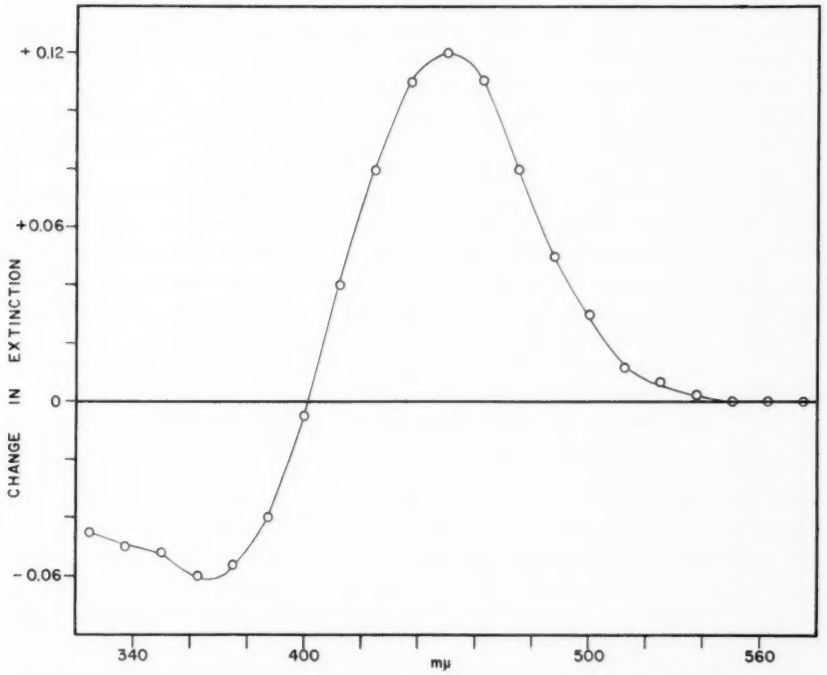


FIG. 3.—Difference spectrum of a photosensitive pigment from the honeybee. An aqueous extract of dark-adapted bees was prepared as indicated in the text. A sample (0.3 ml., pH 6.8) was irradiated for 7 minutes with yellow light (Corning filter No. 3384), and the absorption spectrum was immediately recorded. This was subtracted from the absorption spectrum of the extract before irradiation, to yield the difference spectrum shown. This possesses $\lambda_{max}$ about 450 mμ. The true absorption spectrum of the photopigment has $\lambda_{max}$ somewhat shorter, probably close to 440 mμ.

The identification of retinene proved to be of critical importance in the search for photosensitive pigments in the bee. Because retinene has so far been found only as the chromophore of such pigments, it seemed reasonable to assume that it plays a similar role in the bee. However, the extraction of a photosensitive pigment from bees presents special problems. It is possible to estimate the amount of visual pigment present, using as a basis the amount of retinene which can be extracted. Unfortunately, however, the heads of bees contain light-stable pigments in amounts which are vast by comparison. By using the retinene as a marker, it has been possible to devise a fractionation procedure to separate a photosensitive pigment from the bulk of the light-stable contaminants.

Unlike all other visual pigments so far examined, that of the bee is soluble in water without the aid of such solubilizing agents as digitonin. On grinding the

heads of dark-adapted bees in about 10 times their weight of neutral phosphate buffer, about 80 per cent of the total retinene-protein complex is brought into solution. This and all succeeding operations are carried out under dim red light or in complete darkness and at 4° C. The solution is cleared, first, by straining through cheesecloth, then by centrifuging. On bringing the buffer extract to 45 per cent saturation with ammonium sulfate, large amounts of colored contaminants are precipitated, leaving the retinene-protein in solution. The latter is itself precipitated by 60 per cent saturation with ammonium sulfate. The precipitate is redissolved in fresh buffer, and small molecular-weight impurities are removed by dialysis. Further colored contaminants are precipitated with 25 volumes per cent ethanol at −12° C., again leaving the retinene-protein in solution.

Using these techniques, the extract from 20 gm. of heads is concentrated in about 1 ml. of a partially purified solution, which, in favorable extracts, contains nearly 50 per cent of the retinene originally obtainable from the heads. On exposure to light, this solution exhibits a partial bleaching, owing to the presence of a photosensitive pigment with $\lambda_{max}$ about 440 m$\mu$ (Fig. 3). The change in extinction in the region 440–450 m$\mu$ is roughly equivalent to the retinene formed by bleaching, if it is assumed that the photopigment possesses an extinction per retinene residue like that of rhodopsin, about 40,000.

It is not yet clear whether this photopigment comes from the compound eyes, the ocelli, or both. Possibly it is only one of several photosensitive pigments in the bee, an insect alleged to have color vision (see, for example, von Frisch[7] and Daumer[8]). Preliminary observations suggest that this pigment is formed from the neo-b isomer of retinene, and further work is now in progress to elaborate this and other points. The spectral sensitivity functions of bee compound eyes and ocelli are also being measured. Evidence has been obtained of one such sensitivity function in drones, maximal at about 440 m$\mu$, which may be based upon the photopigment here described.

* This research was supported in part by grants to Professor George Wald from the Office for Naval Research and the Rockefeller Foundation.

† This investigation was carried out during the tenure of a fellowship (BF-6302-C) from the Institute of Neurological Diseases and Blindness, Public Health Service. I would also like to thank Professor George Wald for his helpful criticism and advice.

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[3] J. J. Wolken, *Trans. N.Y. Acad. Sci.*, Ser. II, **19**, 315, 1957.

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# *THE LUCIFERIN-LUCIFERASE REACTION IN A FISH, PARAPRIACANTHUS BERYCIFORMIS, OF NEWLY DISCOVERED LUMINESCENCE**

By Yata Haneda and Frank H. Johnson

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Although bioluminescence, in general, presumably depends on the presence of a specific enzyme-substrate system, extracts of heat-labile ("luciferase") and heat-stable ("luciferin") components which emit light when mixed (the luciferin-luciferase reaction) have been definitely obtained from only seven[1] of the many types of luminescent organisms known, viz., certain bivalves (*Pholas, Rocellaria*), ostracod crustacea (*Cypridina*), the marine fireworm (*Odontosyllis*), deep-sea shrimp (*Systellaspis, Heterocarpus*, the fresh-water limpet (*Latia*), fireflies, and bacteria. The one instance in which a luciferin-luciferase reaction has been reported in products of a luminous fish (*Malacocephalus laevis*)[2] is questionable because of the bacterial-like properties of the material and the subsequent cultivation of luminous bacteria from the light organ.[3]

Luminescence was recently detected (Y. H.) in specimens of a small fish, *Parapriacanthus beryciformis* Franz,[4] in a Shimoda fish market. Common to coastal waters of Japan from Yokohama southward, this fish of the family Pempheridae presents no unusual features externally, and close scrutiny of living specimens at night is needed to observe their luminescence. Internally, two photogenic ducts occur ventrally between the pectoral fins and anus (Figs. 1 and 2) underlying translucent muscle which serves as a lens, as in *Acropoma japonicum, Paratrachichthys prosthemius*, and several fish of the Leiognathidae.[5] Of the two ducts, the more anterior, or thoracic, duct is a V-shaped, thin, white, tubular structure, closed at each end; in appearance it resembles the U-shaped duct of *Acropoma japonicum* that contains symbiotic luminous bacteria. In a fish of 75 mm. in total length, this duct is approximately 10 mm. long, 1 mm. wide, and 0.2 mm. thick. The more posterior, or anal, duct is filiform, lying mid-ventrally and extending to the rectum, with which it communicates through a small pore. In a 75-mm.-long fish, this duct is about 7 mm. long, 1 mm. wide, and 0.2 mm. thick. By pressing a living specimen between one's fingers, a luminous exudate is forced out of the anus. Attempts to cultivate luminous bacteria, by the usual methods, from the photogenic organs of ten specimens gave negative results.

When dissected out, minced, and placed in deionized or tap water, the photogenic organs yield a brightly luminous solution whose light persists for many hours. Luminescence of such solutions is oxygen-dependent, for it is extinguished by evacuating the air and returns when air is readmitted.

Preparations of crude luciferase were made in two ways from the luminescent ducts dissected out of about a dozen specimens in each case. The first method consisted merely of grinding the ducts in a mortar and working up the resulting

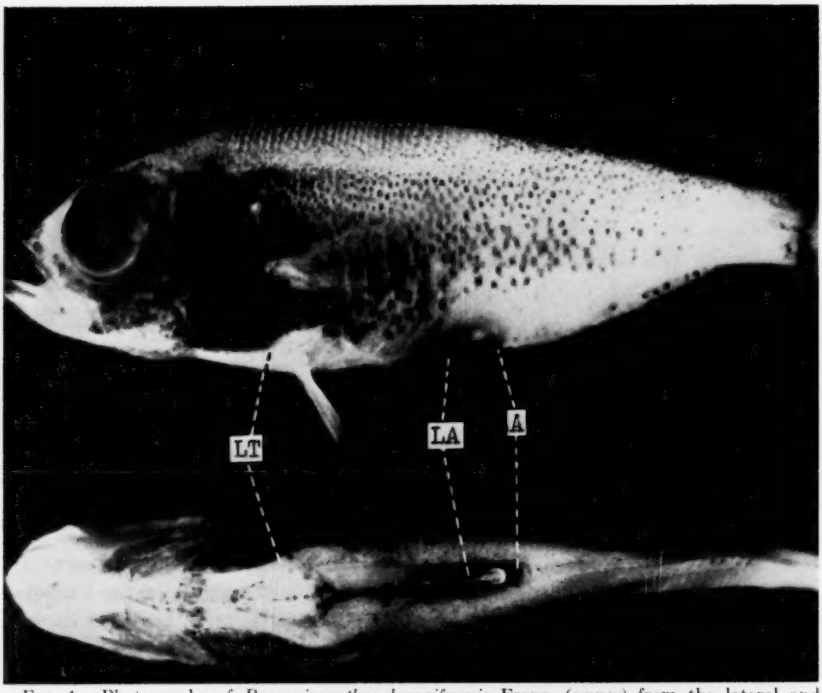


FIG. 1.—Photographs of *Parapriacanthus beryciformis* Franz, (*upper*) from the lateral and (*lower*) from the ventral aspects, respectively, showing the position of the luminous organs. *LT*, thoracic luminous duct; *LA*, anal luminous duct; *A*, anus.

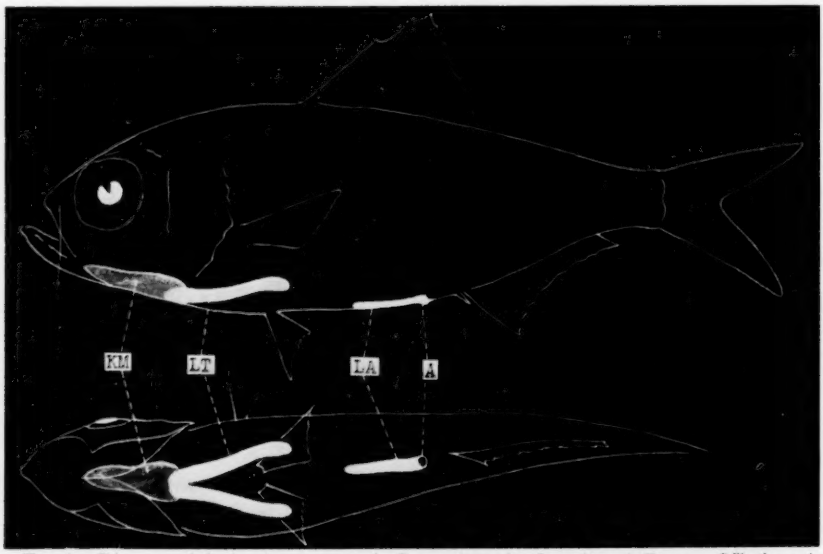


FIG. 2.—Diagram of the luminous organs in *Parapriacanthus beryciformis* Franz. *LT*, thoracic luminous duct; *LA*, anal luminous duct; *KM*, keel muscle; *A*, anus.

brei in sea water or in deionized water. The second consisted of placing the minced ducts in a large volume of cold acetone, followed by washing with acetone at room temperature, grinding in a mortar, and taking up in sea water. This latter method resulted in a completely dark solution, whereas at least a dim light persisted for a long time in solutions prepared by the former method. Preparations of crude luciferin were made by boiling for 1–2 minutes a similar quantity of minced ducts in a few milliliters of deionized water, followed by rapid cooling in an ice bath. Very brief boiling was not sufficient to extinguish the light after rapid cooling.

Luminescence immediately appeared on adding the crude luciferin solution to the acetone-extracted enzyme preparation; when added to faintly luminous preparations of the organs that had been merely ground, the light immediately became bright. Hot- and cold-water extracts prepared from non-luminous tissues from various parts of the body by boiling and grinding, respectively, did not cross-react to give luminescence with extracts from the luminous organs, but extracts prepared from the two luminous organs separately reacted with each other.

Adenosine triphosphate, important in the luminescence of firefly extracts[6] had no observable effect on the luminescence of the above extracts. Accessory factors that influence the luminescence of bacterial extracts, including flavine mononucleotide (FMN), reduced diphosphopyridine nucleotide (DPNH), and decaldehyde,[7] singly or in combinations of FMN plus DPNH, and FMN plus DPNH plus decaldehyde, had only the effect that a slightly brighter luminescence was evident when DPNH was added, either alone or in the above combinations.

The first clear demonstration of a luciferin-luciferase reaction in the luminescence of a fish is of special interest in extending the small list of bioluminescent organisms from which components active in light emission have been successfully separated and invites further biochemical research on this system, as well as a re-examination and extension of investigations of other luminous fish. The histology of the luminous organs is currently under study.

We wish to express our sincere thanks to Dr. T. Abe, who identified the specimens, and to Dr. S. Takatsuki, director of the Shimoda Marine Biological Station, who assisted us in numerous ways during our stay in Shimoda.

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[3] Y. Haneda, *Jap. J. Physiol.*, **3,** 219–326, 1938; *J. Med. Sci.*, III, *Biophys.*, **5,** 355–366, 1938.

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# *THE ACTION OF LIGHT ON RHODOPSIN**

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*Communicated by George Wald, December 30, 1957*

Rhodopsin, a red visual pigment of vertebrate rods, is composed of the yellow carotenoid derivative, retinene, joined to the colorless protein, opsin. It is synthesized by the combination of opsin with a specific hindered *cis* isomer of retinene, neo-*b* (11-*cis*) and bleaches in the light to a mixture of opsin and all-*trans* retinene (cf. Fig. 1). Another *cis* isomer of retinene—iso-*a* (9-*cis*)—combines with opsin to form a second photosensitive pigment, isorhodopsin. Isorhodopsin differs spectroscopically from rhodopsin but bleaches in the light to the identical mixture of all-*trans* retinene and opsin. The synthesis and bleaching of these pigments therefore involve cycles of stereoisomerization of retinene.[1] This seems to be a general situation that involves all the known visual pigments—rhodopsin, iodopsin, porphyropsin, and cyanopsin—and the corresponding isopigments.[2, 3]

If rhodopsin or isorhodopsin is bleached with heat instead of light, the retinene is released in its original *cis* configuration.[4] Stereoisomerization to the all-*trans* configuration is therefore associated not with all bleaching but specifically with bleaching by light.[5]

It has been known for some years that, in the light, rhodopsin bleaches in stages, over intermediates which are short-lived at room temperature, yet stable at low temperatures or in the absence of water.[6, 7] Light alone does not bleach (i.e., decolorize) rhodopsin but converts it to the red photoproduct, lumi-rhodopsin. Lumi-rhodopsin is stable below about $-45°$ C. Above this temperature, in light or darkness, it goes over to a second orange-red compound, called meta-rhodopsin. In lumi- and meta-rhodopsin the chromophore is still attached to opsin. The major color shift—i.e., bleaching—accompanies the hydrolysis of meta-rhodopsin to retinene and opsin. In light or darkness, vertebrate meta-rhodopsins hydrolyze above about $-15°$ C.,[7] squid and lobster meta-rhodopsins above about $+20°$ C.[8, 9] The slow course of this latter reaction strongly suggests that visual excitation precedes the release of retinene and depends upon the light reaction itself or, at most, on the conversion of lumi-rhodopsin to meta-rhodopsin.[10]

On warming meta-rhodopsin in the dark, one obtains a mixture containing roughly one part of retinene and opsin and one part of rhodopsin and isorhodopsin.[7, 11] It was assumed earlier that all these products were formed in the dark, i.e., that meta-rhodopsin in the dark was converted back to rhodopsin and isorhodopsin, in addition to being degraded to retinene and opsin.[7, 11] Recent experiments, however, have clarified the mechanism of the light reaction and necessitate a reinterpretation of these observations.

*Squid rhodopsin.*—Squid rhodopsin does not usually bleach in the light, owing to the relative stability of squid meta-rhodopsin (see above). This enables one to examine squid meta-rhodopsin under more favorable conditions than vertebrate meta-rhodopsins. Thus we have found that, whereas the chromophore of squid rhodopsin, like vertebrate rhodopsin, has the neo-*b* (11-*cis*) configuration, that of meta-rhodopsin is all-*trans*. Thermal bleaching, which does not isomerize the

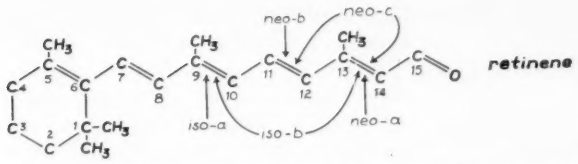



FIG. 1.—Structural formula of retinene, showing the system of numbering carbon atoms. The structure as shown has the all-*trans* configuration. Arrows indicate the double bonds which are in *cis* configuration in the various *cis* isomers.

chromophore (see above), releases predominantly neo-*b* retinene from rhodopsin, but all-*trans* retinene from meta-rhodopsin (Fig. 2).

This is readily demonstrated by irradiating the products of thermal bleaching with white light, which isomerizes retinene to a steady-state mixture of isomers.[1, 12] As shown in Figure 2, the retinene derived from rhodopsin rises in extinction, and $\lambda_{max}$ shifts to longer wave lengths, indicating an isomerization predominantly from *cis* to *trans*, whereas the retinene derived from meta-rhodopsin exhibits the opposite behavior. The retinene released by rhodopsin has been further identified as the neo-*b* isomer by its capacity to form rhodopsin upon addition of cattle opsin. The conversion of rhodopsin to meta-rhodopsin therefore involves the isomerization of the chromophore from the neo-*b* to the all-*trans* configuration.

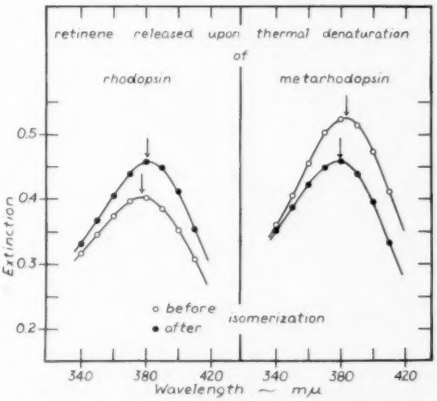



FIG. 2.—Stereoisomeric configurations of retinene released by thermal bleaching of squid rhodopsin (*left*) and meta-rhodopsin (*right*) in 2 per cent digitonin, pH 6.3. To minimize thermal isomerization of the products of bleaching (see n. 5), rhodopsin and meta-rhodopsin are denatured by immersing them in a 70° water bath for 30 seconds, and the products chilled immediately on ice. On irradiation with a white isomerizing light (160-watt tungsten-filament lamp with Corning filters 3060 + 3966), the retinene liberated from rhodopsin rises in extinction and $\lambda_{max}$ shifts to longer wave lengths (*left*), whereas the retinene liberated from meta-rhodopsin exhibits opposite changes (*right*). The results are the same whether acid or alkaline meta-rhodopsin is used.

Squid meta-rhodopsin is a pH indicator, red in neutral and mildly acid solution and yellow in alkaline solution. The interconversion of these two forms proceeds readily in the dark and involves only the reversible dissociation of one hydrogen ion from an acid-binding group with pK 7.7.[13] The two forms, called *acid* and *alkaline meta-rhodopsin*, both contain all-*trans* retinene. Their absorption spectra are shown in Figure 3.

Light, having converted rhodopsin to lumi-rhodopsin, can go on to isomerize the all-*trans* chromophore of lumi- or meta-rhodopsin back to the neo-*b* configuration and thus can regenerate rhodopsin. With acid meta-rhodopsin, the isomerization is stereospecific and yields only rhodopsin. The isomerization of alkaline meta-

rhodopsin is less specific, yielding a mixture of rhodopsin and isorhodopsin. Light therefore promotes the interconversion of rhodopsin and meta-rhodopsin, forming steady-state mixtures of both:[13]

$$\text{Squid rhodopsin} \xleftrightarrow{\text{light}} \text{meta-rhodopsin} \xrightarrow{H_2O,\ >\ 20^\circ\ C.} \text{retinene} + \text{opsin}. \quad (1)$$

The absorption spectra of rhodopsin and acid meta-rhodopsin overlap so widely (cf. Fig. 3) that, starting with either, irradiation with yellow or orange light (Corning filter 3389 or 3482) produces steady-state mixtures containing both pigments in a ratio of about 0.9:1. Since meta-rhodopsin has about 1.5 times the molar extinction of rhodopsin (Fig. 3), the quantum efficiency for isomerizing meta-rhodopsin to rhodopsin (*trans* to neo-*b*) must be about 0.6 (i.e., 0.9/1.5) that of the reverse isomerization.

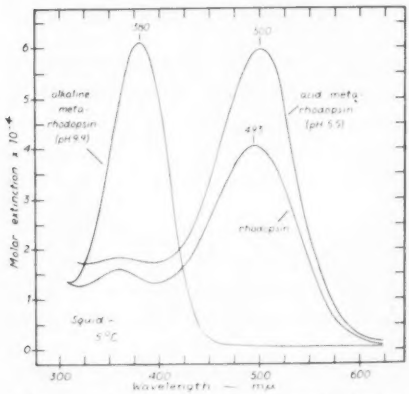



FIG. 3.—Absorption spectra of squid rhodopsin; alkaline meta-rhodopsin produced by irradiating rhodopsin with orange light at pH 9.9; and acid meta-rhodopsin produced by acidification of alkaline meta-rhodopsin to pH 5.5 in the dark. Solvent: 2 per cent aqueous digitonin. For detailed procedure, see n. 13.

Alkaline meta-rhodopsin does not absorb yellow or orange light (cf. Fig. 3) and hence is not isomerized by it. By irradiation with yellow or orange light, squid rhodopsin can therefore be converted quantitatively to alkaline meta-rhodopsin. By acidifying this in the dark, one obtains pure acid meta-rhodopsin—the only way to prepare the pure pigment.[13]

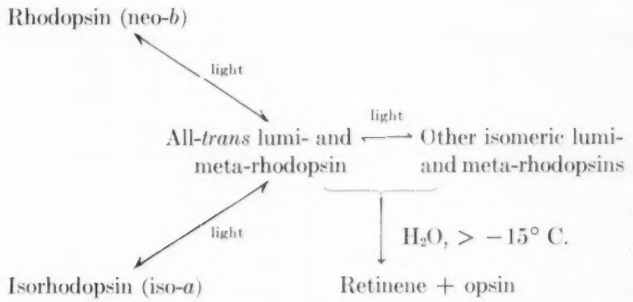
It has been known for some time that retinene in solution is isomerized by simple exposure to light.[1, 12] We see now that light also isomerizes retinene while attached to opsin as the chromophore of squid rhodopsin or meta-rhodopsin. Is this also true for vertebrate rhodopsins?

*Cattle rhodopsin.*—As mentioned above, cattle or frog rhodopsin, after exhaustive irradiation in the cold or in dry films, yields in the dark about half the final product in the form of regenerated rhodopsin and isorhodopsin.[7, 11] It had been assumed earlier that these pigments were generated by a thermal ("dark") back-reaction from lumi- or meta-rhodopsin. Our experience with squid rhodopsin, however, suggests the following reinterpretation.

The first effect of light upon rhodopsin is to form all-*trans* lumi-rhodopsin, which goes over to all-*trans* meta-rhodopsin by a thermal reaction. Light can go on to isomerize the chromophore of either compound to a steady-state mixture of geometrical isomers. The neo-*b* component of this mixture *is* rhodopsin; the iso-*a* component *is* isorhodopsin. No thermal back-reaction is needed to make these substances, for they are regenerated by isomerization *in the light.*

The remaining isomers—all-*trans*, neo-*a*, and so on (cf. Fig. 1)—constitute an isomeric set of lumi- or meta-rhodopsins, all of which are unstable when warmed

in the presence of water and hence bleach to mixtures of retinene and opsin. The reaction sequence can therefore be summarized as follows:




(2)

That this is the true situation is demonstrated by the following experiment (Fig. 4). Rhodopsin at −17° C. (curve *1*) is irradiated exhaustively with white light. Since lumi-rhodopsin is unstable above about −45° C., this yields a product (curve *2*) which is identical with what has previously been called "meta-rhodopsin." Its heterogeneity can be demonstrated by bringing it to about pH 13 in the dark, without raising the temperature. Under these conditions, rhodopsin and isorhodopsin bleach slowly (half-lives of about 12 and 5 hours, respectively), and their destruction can be fitted by simple monomolecular decay curves. The product of irradiation, however, does not bleach in this simple manner. The course of its decomposition can be fitted by combining the decay curves of rhodopsin and isorhodopsin with the monomolecular decay curve of a third, much less stable, component with a half-life of about 15 minutes.[14] At the end of 3 hours at pH 13, the solution contains roughly two parts retinene and opsin and one part rhodopsin and isorhodopsin (curve *3*). The presence of rhodopsin and isorhodopsin under these rigorous conditions (pH 13, −17° C.) strongly suggests their photochemical origin.

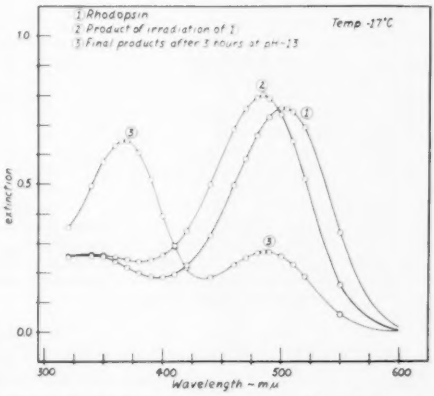



FIG. 4.—Action of light on cattle rhodopsin. Cattle rhodopsin at −17° C. (spectrum *1*) is irradiated with white light (tungsten-filament lamp, with Jena BG 18 heat filter) until the spectrum stops changing. The product of irradiation (spectrum *2*) is not a homogeneous compound, as is shown by raising the pH in the dark from 7 to about 13 with 9 *N* sodium hydroxide. This decomposes part of the mixture. After 3 hours, the solution contains about two parts retinene and opsin, and one part rhodopsin and isorhodopsin (spectrum *3*). Solvent: 0.5 per cent digitonin in a 1:1 glycerol-water mixture.

The terms "lumi-" and "meta-rhodopsin," as used previously,[7] refer to the product of irradiation (cf. curve *2*), hence not to single compounds but to steady-state

mixtures of chromoproteins including photoregenerated rhodopsin and isorhodopsin. As can be seen from equation (2), we are now redefining lumi- and meta-rhodopsin as the *labile* fraction of the mixture of stereoisomeric chromoproteins, the fraction which hydrolyzes to retinene and opsin above about $-15°$ C. in the dark. The difference spectrum of cattle meta-rhodopsin so defined is shown in Figure 5.

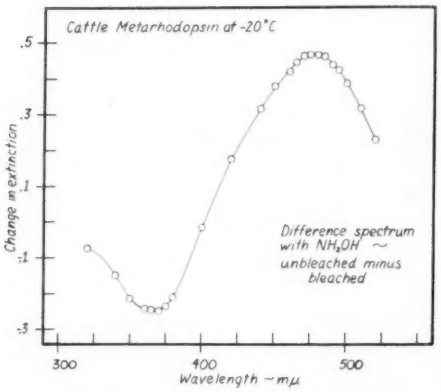



FIG. 5.—Difference spectrum of cattle meta-rhodopsin at $-20°$ C. Solvent: 0.3 per cent digitonin in a 2:1 glycerol-water mixture. Rhodopsin at $-20°$ C. is irradiated exhaustively with monochromatic light at 500 mμ. Hydroxylamine is then added to a final concentration of about 0.1 *M* and the spectrum measured. The solution is now warmed to room temperature in the dark. This bleaches the meta-rhodopsin, converting its chromophore to retinene oxime. After about $^3/_4$ hour, the solution is recooled to $-20°$ C., and the spectrum is measured again. The difference between the two is shown. Since retinene oxime has essentially no absorption at wave lengths longer than about 440 mμ, above this wave length the difference spectrum is identical with the absorption spectrum.

As one would expect on the basis of this formulation, the experiment shown in Figure 4 yields the same mixtures (spectra *2* and *3*), whether one starts with rhodopsin or with isorhodopsin (viz., eq. [2]).

We have pursued the photochemical relationships somewhat further. As can be seen from Table 1, the absorption maxima of rhodopsin, isorhodopsin, and metarhodopsin lie at progressively shorter wave lengths. The steady-state mixtures of these compounds should therefore vary somewhat in composition, depending on the wave length of light used to irradiate them. Thus green light, absorbed preferentially by rhodopsin, should favor the formation of meta-rhodopsin, whereas blue light, absorbed preferentially by meta-rhodopsin, would produce a steady-state mixture containing more rhodopsin. Analyses of steady-state mixtures produced by irradiation at 450, 500, and 550 mμ are shown in Figure 6 and Table 2. As predicted, the mixture produced by irradiation at 550 mμ contains about seven times as much meta-rhodopsin as rhodopsin, whereas irradiation at 450 mμ yields more nearly equal amounts of the two pigments. The mixture produced at 500 mμ is intermediate in composition. The absolute concentration of isorhodopsin is almost the same at all three wave lengths, but this is fortuitous.

From these data one can calculate the relative quantum efficiencies ($\gamma$)[16] for the conversion of meta-rhodopsin to rhodopsin and isorhodopsin. The steady-state concentrations of rhodopsin, meta-rhodopsin, and isorhodopsin following irradiation at each wave length are given by the following relationships:[17]

$$\text{(i)}\quad \frac{(M)}{(R)} = \frac{\epsilon_r\,\gamma_{r\rightarrow m}}{\epsilon_m\,\gamma_{m\rightarrow r}} \text{ and (ii) } \frac{(M)}{(I)} = \frac{\epsilon_i\,\gamma_{i\rightarrow m}}{\epsilon_m\,\gamma_{m\rightarrow i}},$$

in which $(R)$, $(M)$ and $(I)$ are the concentrations of rhodopsin, meta-rhodopsin, and isorhodopsin; $\epsilon_r$, $\epsilon_m$, and $\epsilon_i$, their molar extinctions at the wave length of irradiation;

and $\gamma_{r \to m}$, $\gamma_{m \to r}$, $\gamma_{i \to m}$, and $\gamma_{m \to i}$, the quantum efficiencies for their interconversions in the directions indicated by the subscripts.

The molar extinction coefficients of the three compounds are given in Table 3, and their concentrations at the steady state are shown in Figure 6 and Table 2. All quantum efficiencies are expressed relative to the quantum efficiency for converting rhodopsin to meta-rhodopsin ($\gamma_{r \to m}$), arbitrarily taken as 1.

TABLE 1*

ABSORPTION PROPERTIES OF CATTLE RHODOPSIN, ISORHODOPSIN, AND META-RHODOPSIN AT −20° C. IN 2:1 GLYCEROL-WATER MIXTURE CONTAINING 0.3 PER CENT DIGITONIN

| COMPOUND | $\lambda_{max}$ (mμ) | MOLAR EXTINCTION AT $\lambda_{max}$ |
|---|---|---|
| Rhodopsin | 502 | 44,000 |
| Isorhodopsin | 490 | 47,700 |
| Meta-rhodopsin | 478 ± 2 | 48,500 ± 2,000 |

* The molar extinctions of rhodopsin and isorhodopsin are calculated from the molar extinctions in this solvent system at room temperature (42,000 and 43,000) by cooling solutions of known extinction to −20° C. and remeasuring their absorption spectra. The data for meta-rhodopsin, averaged from the three experiments shown in Fig. 6, are taken from the difference spectra obtained by subtracting spectra *3* from spectra *2*.

TABLE 2*

COMPOSITIONS OF STEADY-STATE MIXTURE PRODUCED BY IRRADIATING RHODOPSIN AT −20° C. WITH MONOCHROMATIC LIGHTS OF VARIOUS WAVE LENGTHS (HALF-WIDTH = 11 mμ)

| | STEADY-STATE CONCENTRATIONS FOLLOWING IRRADIATION AT | | |
|---|---|---|---|
| COMPOUND | 450 mμ | 500 mμ | 550 mμ |
| Rhodopsin | 34 | 25 | 11 |
| Isorhodopsin | 17 | 17 | 14 |
| Meta-rhodopsin | 49 | 58 | 75 |

* Concentrations, given in percentages, are calculated from the data shown in Fig. 6: rhodopsin and isorhodopsin from spectra *3*, by a method similar in principle to one previously described (see R. Hubbard, *J. Gen. Physiol.*, **39**, 935, 1955–56); meta-rhodopsin from the differences between the initial concentration of rhodopsin (spectrum *1*) and the final concentrations of rhodopsin and isorhodopsin (spectra *3*).

TABLE 3*

MOLAR EXTINCTION COEFFICIENTS (ε) OF CATTLE RHODOPSIN, ISORHODOPSIN, AND META-RHODOPSIN AT 450, 500, AND 550 mμ.

| | MOLAR EXTINCTIONS AT | | |
|---|---|---|---|
| COMPOUND | 450 mμ | 500 mμ | 550 mμ |
| Rhodopsin | 21,800 | 43,900 | 18,600 |
| Isorhodopsin | 31,100 | 45,800 | 9,080 |
| Meta-rhodopsin | 35,800 | 40,600 | 5,400 |

* Conditions as described in Table 1.

We can calculate $\gamma_{m \to r}$ from equation (i). In order to calculate $\gamma_{m \to i}$, however, we need to know $\gamma_{i \to m}$. This is determined as follows: Rhodopsin and isorhodopsin are bleached with dim white light under conditions in which the light reaction is limiting. The rate of bleaching of each compound is then governed only by the rate at which its chromophore is isomerized to the all-*trans* configuration, as shown in equation (2). Rhodopsin and isorhodopsin absorb white light to about the same extent (within 10 per cent). Under these conditions, their relative rates of bleaching therefore reflect the relative quantum efficiencies for their conversion to all-*trans* meta-rhodopsin. Thus, as seen in Figure 7, we find that the conversion

of isorhodopsin to meta-rhodopsin has a relative quantum efficiency of 0.3. With this value for $\gamma_{i \rightarrow m}$ we can calculate $\gamma_{m \rightarrow i}$ from equation (ii).

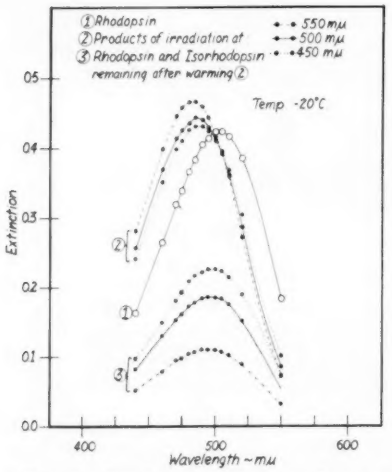



FIG. 6.—Steady-state mixtures of meta-rhodopsin, rhodopsin, and isorhodopsin produced by irradiating cattle rhodopsin at −20° C. with monochromatic lights of 450, 500, and 550 mμ (half-width = 11 mμ). Rhodopsin (spectrum *1*) is irradiated until there is no further change in spectrum (spectra *2*). Hydroxylamine is now added to a final concentration of about 0.1 *M*, and the solutions are warmed to room temperature in the dark to convert meta-rhodopsin to retinene oxime. The solutions are then recooled to −20° C. and the spectra remeasured and corrected for dilution by hydroxylamine (spectra *3*). The three conditions of irradiation yield different mixtures (spectra *2*) containing different amounts of rhodopsin and isorhodopsin (spectra *3*). The analyses of the mixtures are summarized in Table 2. Solvents as in Fig. 5. These experiments will be described in more detail elsewhere (A. Kropf, in preparation).

The results of these calculations are shown in Table 4. They show that the quantum efficiency for converting rhodopsin to meta-rhodopsin is twice that of the reverse reaction (1:0.5), and the quantum efficiency for converting isorhodopsin to meta-rhodopsin about three times that of the reverse reaction (0.3:0.09).[18]

These experiments have dealt primarily with meta-rhodopsin, although light, in fact, converts rhodopsin to lumi-rhodopsin. What is the difference between lumi- and meta-rhodopsin? Lumi-rhodopsin is stable below about −45° C. and seems to make a fleeting appearance when rhodopsin or isorhodopsin is irradiated at −20° C. It is therefore reasonable to assume that lumi-rhodopsin is the initial product of irradiating rhodopsin also at higher temperatures. Its chromophore apparently has the same isomeric configuration as that of meta-rhodopsin, and lumi-rhodopsin can be re-isomerized to rhodopsin and isorhodopsin. The thermal ("dark") reaction which converts lumi-rhodopsin to meta-rhodopsin probably involves a rearrangement of the opsin or of the binding of retinene to opsin, for it does not affect the stereoisomeric configuration of the chromophore.

*Discussion.*—Bleaching, though initiated by the photoisomerization of the

TABLE 4*

RELATIVE QUANTUM EFFICIENCIES (γ) FOR CONVERTING CATTLE META-RHODOPSIN TO RHODOPSIN AND ISORHODOPSIN BY IRRADIATION AT 450, 500, AND 550 mμ

| WAVE LENGTH OF IRRADIATION (mμ) | RELATIVE QUANTUM EFFICIENCIES FOR REACTIONS Meta-Rhodopsin to Rhodopsin | Meta-Rhodopsin to Isorhodopsin |
|---|---|---|
| 450 | 0.4 | 0.09 |
| 500 | 0.5 | 0.1 |
| 550 | 0.5 | 0.09 |
| Averages | 0.5 | 0.09 |

* Calculated from the data presented in Fig. 6 and Table 2, by means of equations (i) and (ii), as described in the text. The values obtained at the three wave lengths are averaged in the bottom line. However, it remains to be demonstrated whether these quantum efficiencies are independent of wave length and whether it is therefore legitimate to average them.

rhodopsin chromophore to the all-*trans* configuration, is actually effected by a "dark" reaction—the hydrolysis of meta-rhodopsin to retinene and opsin. Light can therefore enter the reaction a second time and *inhibit* bleaching by isomerizing lumi- or meta-rhodopsin back to rhodopsin and isorhodopsin. Thus light, once it has triggered the bleaching of rhodopsin, can go on to reverse this effect.

It has been assumed until now that the quantum efficiency for bleaching rhodopsin, as for all simple photochemical reactions, is independent of light intensity and temperature. This is true at relatively low light intensities.[19] However, since the bleaching of rhodopsin is *not* a simple photochemical reaction, its quantum efficiency cannot under all conditions be that of the initial light reaction—the formation of lumi-rhodopsin. It must involve the probability of further quanta interfering with bleaching by re-isomerizing the chromophores of lumi- or meta-rhodopsin. Obviously, if the rate of delivering quanta (i.e., the light intensity) is so low that meta-rhodopsin is hydrolyzed to retinene and opsin before it can absorb quanta, the quantum efficiency of bleaching will be identical with that of the initial light reaction. However, if the light intensity is increased or the rate of hydrolysis of meta-rhodopsin decreased to the point at which lumi- or meta-rhodopsin begin to absorb quanta, the quantum efficiency of bleaching should decrease. We hope shortly to define the limiting conditions for these effects.

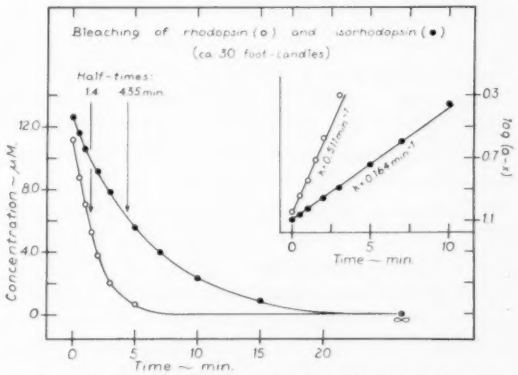



FIG. 7.—Kinetics of photochemical bleaching of cattle rhodopsin and isorhodopsin at 25° C. Solvent: 1.6 per cent aqueous digitonin, 0.2 *M* in hydroxylamine. pH 6.3. The concentrations of rhodopsin and isorhodopsin are plotted as functions of the time of irradiation with dim white light (about 30 foot-candles). In the insert, the same data are replotted in terms of the equation for a monomolecular reaction. Although bleaching involves light and thermal ("dark") reactions, the conditions of this experiment are such that the rate of bleaching is limited by the light reaction. The rate constants ($k$) show that, under identical conditions, rhodopsin bleaches about three times as fast as isorhodopsin.

If vision, as is generally believed, is triggered not by the *bleaching* of rhodopsin but by its conversion to lumi- or meta-rhodopsin, the foregoing considerations are not relevant to the excitation process. That is, the quantum efficiency for vision probably is independent of intensity.

Thermal mechanisms for regenerating rhodopsin[15, 20] probably keep pace with its photodecomposition in dim light. In bright light, however, photoregeneration from lumi- and meta-rhodopsin may serve as an additional source of rhodopsin. That a sufficient number of quanta, if delivered within the lifetimes of lumi- and meta-rhodopsin, produce the photosteady state in vivo, is suggested by Hagins's observation that in a rabbit's eye a photoflash, "no matter how bright, so long as it lasted less than 1 msec . . . could bleach no more than half the rhodopsin in an

illuminated area."[21] However, it remains to be established at what intensities this mechanism is activated and hence whether it has physiological significance.

These considerations lead one to wonder why isorhodopsin does not accumulate in the eye.[2] There is no reason to doubt that isorhodopsin will be formed at light intensities which are sufficiently high to support photoregeneration. However, any isorhodopsin which may accumulate during an exposure to bright light will bleach under ordinary conditions of illumination, when the rate of photoregeneration is probably negligible. The only enzymatic isomerization mechanism which has so far been identified yields exclusively the neo-*b* isomer of retinene, hence rhodopsin.[15] We must conclude, therefore, that *in vivo* isorhodopsin will be found only under very special circumstances.

*Summary.*—Rhodopsin is composed of the colorless protein, opsin, combined with the hindered *cis* isomer of retinene, neo-*b* (11-*cis*). Light isomerizes the neo-*b* chromophore, while still attached to opsin, to the all-*trans* configuration, thereby converting rhodopsin to all-*trans* lumi- and meta-rhodopsin. This is probably the reaction responsible for visual excitation. Meta-rhodopsin from lobster and squid is stable below about 20° C., and vertebrate meta-rhodopsins below about −15° C. Above these temperatures, the meta-rhodopsins hydrolyze to retinene and opsin, the reaction responsible for bleaching.

Light, having converted rhodopsin to all-*trans* lumi-rhodopsin, can go on to isomerize the all-*trans* chromophores of lumi- or meta-rhodopsin to steady-state mixtures of stereoisomeric chromophores, all still attached to opsin. The neo-*b* fraction of this mixture constitutes rhodopsin, the iso-*a* fraction isorhodopsin. The other isomers form stereoisomeric sets of lumi- and meta-rhodopsins, all of which hydrolyze in the dark to retinene and opsin. Thus, though it was thought previously that rhodopsin and isorhodopsin were regenerated from meta-rhodopsin by a "dark" reaction, they are, in fact, produced by the isomerization of the chromophores of lumi- and meta-rhodopsin *by light*.

Since light therefore induces not only the bleaching of rhodopsin but also its regeneration, the quantum efficiency of bleaching should decrease under all conditions which favor the photoregeneration of rhodopsin from lumi- or meta-rhodopsin. The quantum efficiency of vision, however, probably depends only on the initial photoisomerization of rhodopsin to all-*trans* lumi-rhodopsin and should not be affected by the photochemical back-reactions.

* This research has been supported in part by grants to Professor George Wald from the U.S. Public Health Service (B-568, C-3,4) and the Rockefeller Foundation and by a fellowship to one of us (A. K.) from the American Cancer Society. We wish to thank Professor Wald for many suggestions and discussions. We should also like to express our indebtedness to Mr. Paul K. Brown for having turned over to one of us (A. K.) his equipment for working with rhodopsin at low temperatures.

[1] R. Hubbard and G. Wald, *J. Gen. Physiol.*, **36**, 269, 1952–53.

[2] There is no evidence that iso-pigments occur in vivo.

[3] G. Wald, P. K. Brown, and P. H. Smith, *Science*, **118**, 505, 1953; *J. Gen. Physiol.*, **38**, 623, 1954–55; G. Wald, *Am. J. Ophthalmol.*, **40**, No. 5, 18, 1955.

[4] R. Hubbard, *Nature* (in press).

[5] It has been stated elsewhere (see n. 1) that chemical bleaching of rhodopsin liberates predominantly all-*trans* retinene. Recent experiments in which rhodopsin was bleached with heat show that this is not always the case (see n. 4). Moreover, when determining the configuration of the initial product of bleaching, one must prevent its isomerization after its release from the

chromophore. For example, neo-*b* retinene, liberated by thermal bleaching, isomerizes at the temperatures required for the denaturation of rhodopsin. In order to ascertain its configuration, the rhodopsin must be denatured rapidly (within about 30 seconds) and the product chilled immediately.

[6] R. J. Lythgoe and J. P. Quilliam, *J. Physiol.*, **94**, 399, 1938; G. Wald, *J. Gen. Physiol.*, **21**, 795, 1937–38; E. E. Broda and C. F. Goodeve, *Proc. Roy. Soc. London, B*, **130**, 217, 1941–42.

[7] G. Wald, J. Durell, and R. C. C. St. George, *Science*, **111**, 179, 1950.

[8] R. C. C. St. George and G. Wald, *Biol. Bull.*, **97**, 248, 1949.

[9] G. Wald and R. Hubbard, *Nature*, **180**, 278, 1957.

[10] Hagins (*Nature*, **177**, 989, 1956) has shown that, following flash photolysis of rhodopsin in a rabbit's eye, yellow or orange compounds—presumably retinene—appear before one can record the electroretinographic response to the flash. He therefore concludes that the release of retinene may be the triggering mechanism, after all. Two facts weaken this inference: (1) the appearance of the electroretinogram itself entails a time lag following excitation, and (2) we now know two organisms—lobster and squid—in which retinene is released much more slowly, if at all, under physiological conditions.

[11] F. D. Collins and R. A. Morton, *Biochem. J.*, **47**, 18, 1950.

[12] R. Hubbard, R. I. Gregerman, and G. Wald, *J. Gen. Physiol.*, **36**, 415, 1952–53.

[13] R. Hubbard and R. C. C. St. George, *J. Gen. Physiol.*, **41**, 501, 1957–58.

[14] These experiments and calculations will be described in more detail elsewhere (A. Kropf, in preparation).

[15] R. Hubbard, *J. Gen. Physiol.*, **39**, 935, 1955–56.

[16] The quantum efficiency ($\gamma$) is defined as the ratio,

$$\frac{\text{Number of molecules reacting}}{\text{Number of quanta absorbed}}.$$

[17] These equations are derived as follows: The rate of a photochemical reaction is given by the product $\gamma I_{abs}$, in which the amount of light absorbed, $I_{abs} = I_0 \epsilon c d$, when $\epsilon c d < 1$. $I_0$ is the intensity of the incident light; $\epsilon$, the molar extinction coefficient of the reacting compound; $c$, its concentration; and $d$, the thickness of the layer of solution which is being irradiated. In a photochemical equilibrium of the type

$$A \xleftarrow{\text{light}} B,$$

the rates of the forward and backward reactions are therefore given by the following expressions:

$$\text{Forward rate} = \gamma_{A \rightarrow B}\, \epsilon_A\, I_0\, (A)\, d, \quad \text{Backward rate} = \gamma_{B \rightarrow A}\, \epsilon_B\, I_0\, (B)\, d,$$

in which $\gamma_{A \rightarrow B}$ and $\gamma_{B \rightarrow A}$ are the quantum efficiencies for the two reactions; $\epsilon_A$ and $\epsilon_B$, the molar extinctions of $A$ and $B$; and $(A)$ and $(B)$, their concentrations. At the photosteady state, the rates of the forward and backward reactions are equal, hence

$$\frac{(B)}{(A)} = \frac{\gamma_{A \rightarrow B}\, \epsilon_A}{\gamma_{B \rightarrow A}\, \epsilon_B}.$$

It should be noted that this formulation treats meta-rhodopsin as though it were a homogeneous compound, which it probably is not (viz. eq. [2]). This may introduce errors; but, since we do not yet have adequate methods with which to analyze meta-rhodopsin into its isomeric components, much less measure their rates of interconversion, we must for the present be content with this approximation.

[18] We should like to draw attention to the fact that the relative rates of isomerization of the three types of chromophores parallel those of the respective isomers of retinene in solution. Thus, upon irradiation, all *trans* retinene yields neo-*b* retinene faster than iso-*a* retinene (see R. Hubbard, *J. Gen. Physiol.*, **39**, 935, 1955–56), and meta-rhodopsin is converted to rhodopsin more rapidly than to isohodopsin. Likewise, neo-*b* retinene is isomerized faster than iso-*a* retinene (see R. Hubbard, *J. Am. Chem. Soc.*, **78**, 4662, 1956), and rhodopsin is converted to meta-rhodopsin more rapidly than is isorhodopsin.

[19] S. Hecht, *J. Gen. Physiol.*, **3**, 286, 1920–21; *ibid.*, **6**, 731, 1923–24. However, Dartnall, Goodeve, and Lythgoe (*Proc. Roy. Soc. London, A*, **164**, 216, 1938) report a somewhat lower quantum yield for bleaching at 5° than at 20°–60° C.

[20] F. D. Collins, J. N. Green, and R. A. Morton, *Biochem. J.*, **53**, 152, 1953; *ibid.*, **56**, 493, 1954.

[21] W. A. Hagins, *J. Physiol.*, **129**, 22P, 1955.

# *ON RESONANCE TRANSFER OF EXCITATION ENERGY BETWEEN AROMATIC AMINOACIDS IN PROTEINS**

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*Communicated November 11, 1957*

Teale and Weber,[1] from studies of absorption and singlet emission spectra for the aromatic amino acids phenylalanine, tyrosine, and tryptophane and from the number of amino acid residues and the dimensions of ordinary globular proteins, point out that internal transfer of excitation energy must occur in proteins. They postulate a transfer between phenylalanine, tyrosine, tryptophane, and heme, the energy being passed from earlier to later members of this series The calculations reported in this paper give not only quantitative corroboration to this concept but emphasize that such a flow of energy should be essentially unidirectional.

In Figure 1 we present the absorption and fluorescent spectra for the aromatic amino acids phenylalanine, tyrosine, and tryptophane and the absorption spectra for reduced diphosphopyridine nucleotide and oxidized riboflavin. An examination of these spectra reveals a considerable overlap of the amino acid emission spectra with their own absorption spectra and with the absorption spectra of reduced diphosphopyridine nucleotide and oxidized riboflavin. The possibility that these spectral overlaps may provide a mechanism for the resonance transfer of excitation energy prompted us to evaluate this possibility quantitatively.

Using the theory of Förster[2] as applied by Karreman and Steele,[3] we have calculated critical distances, $R_0$ (that distance, in Å, between an energy donor and an acceptor molecule over which excitation energy may be transferred by resonance with the same probability that it may be emitted as fluorescence), for the several pair systems between which resonance transfer of energy may occur. Critical distances were calculated with the formula (Förster;[4] Karreman and Steele[3]),

$$R_0 = \sqrt[6]{0.95 \times 10^{-33} \frac{\tau J \tilde{\nu}}{\tilde{\nu}_0^2}},$$

in which $\tau$ is the lifetime of the lowest excited singlet state (calculated from absorption spectra and using the quantum yields reported by Teale and Weber,[1] see Förster[5]), $J$ is the overlap integral and $\tilde{\nu}_0$ is the average wave number between the fluorescent and the absorption maxima of the energy donor molecule. The calculated critical distances, $R_0$ (Å), together with the pertinent parameters, are tabulated in Table 1.

The question arises whether the critical distances calculated will allow a resonance transfer of excitation energy within a protein molecule. The ratio of the three aromatic amino acids within various proteins varies within rather wide limits, but the sum of the three acids is surprisingly constant. In Table 2 we present the ratio of the total number of amino acids to the number of aromatic amino acids in several proteins. Within narrow limits this ratio is close to 12. If the specific weight of

the protein is taken as 1.2 and the average molecular weight of an amino acid as 120, then the protein contains amino acids in a concentration of 10 molar. If every twelfth amino acid is aromatic, then the concentration of aromatic amino acids is approximately 0.8 molar. At this concentration the statistical distance between the aromatic amino acids is of the order of 10 Å. This distance is smaller than the critical distances for the hetero-amino acid pairs, and we might thus anticipate a considerably efficient resonance transfer of excitation energy within protein molecules.

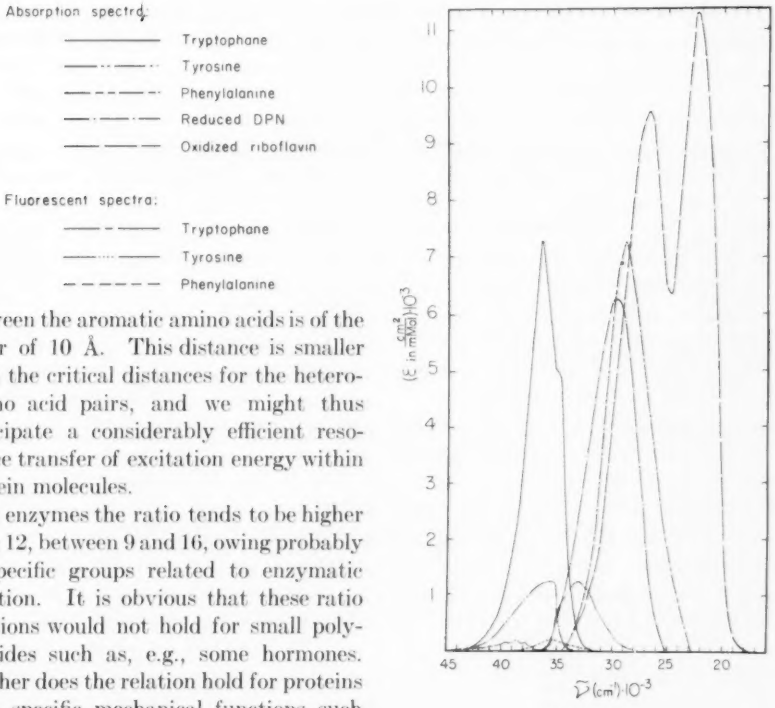


FIG. 1

In enzymes the ratio tends to be higher than 12, between 9 and 16, owing probably to specific groups related to enzymatic function. It is obvious that these ratio relations would not hold for small polypeptides such as, e.g., some hormones. Neither does the relation hold for proteins with specific mechanical functions such as L-meromyosin (ratio = 39).

A significant feature revealed by a study of the spectral data in Figure 1 is that, for any heteromolecular pair combination, an excitation generated in the donor molecule must flow unidirectionally in the direction of lower-excitation states. It is interesting to note that unidirectional flow of excitation energy could occur in two ways: (1) linearly, in the direction of the protein axis, in which case the amino acid sequence and interamino acid residue distances within the protein molecule must be within certain critical values or (2) "three-dimensionally" (as discussed above), in which case the acceptor molecule to which energy is transferred by resonance would be that molecule lying only within a certain critical distance. In both instances coiled or helical structures would impose additional directional specificity to energy flow by constraining the donor and acceptor electronic oscillators in proper alignment for maximum transfer efficiency. From the few published data on proteins with known amino acid sequence, it would be premature to suggest in which way energy might be transferred by resonance within the protein molecule. It is possible that both mechanisms may operate. Estimating

the radii of globular proteins to range between 15 and 30 Å, it can be seen from the reported $R_0$ values that an energy donor molecule can transfer its energy to an acceptor molecule located at almost any point in the molecule with considerable efficiency. In such proteins a certain amino acid sequence would not be a prerequisite for intramolecular energy flow, but in fibrous proteins, where asymmetry is all-important, a specific sequential arrangement of the participating amino acids would be a necessity for a unidirectional resonance transfer of energy along the fiber axis.

TABLE 1

CRITICAL DISTANCES, $R_0$, FOR RESONANCE TRANSFER OF EXCITATION ENERGY BETWEEN GIVEN DONOR AND ACCEPTOR MOLECULAR PAIRS

| Donor-Acceptor Pair | | $\tau \times 10^9$ | $\tau_0 \times 10^{-3}$ | $J\bar{\nu} \times 10^{-8}$ | $R_0$ (Å) |
|---|---|---|---|---|---|
| Phenylalanine | ⇆ phenylalanine | 1.1 | 37.1 | 0.0404 | 5.6* |
| Phenylalanine | → tyrosine | 1.1 | 37.1 | 4.1 | 12.0 |
| Phenylalanine | → tryptophan | 1.1 | 37.1 | 21.1 | 16.0 |
| Tyrosine | ⇄ tyrosine | 0.91 | 34.4 | 0.458 | 8.3* |
| Tyrosine | → tryptophane | 0.91 | 34.4 | 13.0 | 15.0 |
| Tyrosine | → reduced DPN | 0.91 | 34.4 | 341.0 | 25.0 |
| Tyrosine | → oxidized riboflavin | 0.91 | 34.4 | 124.0 | 21.0 |
| Tryptophane | ⇄ tryptophane | 0.20 | 32.6 | 0.326 | 6.3* |
| Tryptophane | → reduced DPN | 0.20 | 32.6 | 1210 | 25.0 |
| Tryptophane | → oxidized riboflavin | 0.20 | 32.6 | 1670 | 26.0 |

* See text for explanation of the parameters (in absolute units). We realize that the assumptions made by Förster (*Ann. d. Phys.*, **2**, 55–75, 1948) may impose considerable uncertainty in the $R_0$ values when $R_0$ is not several times larger than the radii of the interacting molecular species.

TABLE 2*

RATIO OF TOTAL NUMBER OF AMINO ACIDS TO NUMBER OF AROMATIC AMINO ACIDS IN SEVERAL PROTEINS

| Protein | Ratio |
|---|---|
| Horse hemoglobin† | 11 |
| Bovine serum albumin | 13 |
| Human fibrinogen | 11 |
| Human serum albumin | 10 |
| Human globulin | 12 |
| Whole casein | 11 |
| Ovalbumin | 12 |
| Edestin | 13 |
| Conalbumin | 12 |
| Silk fibroin | 12 |
| Gliadin | 13 |
| Zein | 11 |
| Actin | 12 |
| Heavy meromyosin | 12.5 |

* In the construction of this table we calculated for randomly selected proteins the ratio of the total number of amino acids to the total number of aromatic amino acids, based on protein analyses given by G. R. Tristram in *The Proteins*, ed. Hans Neurath and Kenneth Bailey (New York: Academic Press, 1953), **I**, Part A, 181–233. Analyzed data of actin and H. meromyosin were taken from Andrew Szent-Györgyi's review in *Advances in Enzymol.*, **16**, 313, 1955.

† Corresponding to the 4 hemes of the molecule, with a M. W. of 2,400, 22 were subtracted from the total number of amino acid residues in the molecule.

We have included critical distances for the resonance transfer of energy from the amino acids tyrosine and tryptophane to the coenzymes reduced diphosphopyridine nucleotide and oxidized riboflavin because of the potential significance that this interaction may be found to have in energy transformations (e.g., oxidative phosphorylations) and/or energy transfer (conduction). The high $R_0$ values

calculated for these interactions would provide an efficient intermolecular transfer of excitation energy. As regards energy transfer, it might be noted that the results of this paper and the paper by Karreman and Steele[3] provide a "path" for resonance transfer of excitation energy from phenylalanine → tyrosine → tryptophane → reduced diphosphopyridine nucleotide → oxidized riboflavin → cytochromes. Further, this flow should be unidirectional and, within the respiratory components, should proceed in the same direction that electrons move during mitochondrial respiration.

A feature of resonance transfer of excitation energy of obvious importance is that the donor and acceptor molecules may be separated by considerable distances. This fact would eliminate the dilemma of "collisional-orientation" of iron-iron residues for intercytochrome energy transfer and, further, make unnecessary surface-surface interaction specificity other than perhaps electronic oscillator alignment. In this regard, e.g., the iron-heme moiety of cytochrome c, considered by Ehrenberg and Theorell[6] to be localized subsurfacely in the protein matrix, would be unavailable for collisional contact but readily accessible by resonance transfer. Mitochondrial rigidity would maintain the proper donor-acceptor sequence and electronic oscillator alignment. This mechanism, however, still leaves unanswered the details of electron transfer per se, with its probable interaction specificity.

In conclusion it should be pointed out that the observations reported in this paper refer to singlet excitation states of reacting molecules, while the research of this institute suggests that triplet excitation states may play the major role in energy exchanges and transformations. Though we have obtained the triplet emission spectra for the aromatic amino acids, the as yet unavailable triplet absorption data for these amino acids preclude our calculating resonance transfer data for the triplet excitation states. Nonetheless, it should be emphasized that Terenin and Ermolaev[7] have presented experimental evidence for a resonance transfer of triplet state energy.

*Summary.*—Critical distances, $R_0$, have been calculated for the resonance transfer of singlet excitation energy between the aromatic amino acids phenylalanine, tyrosine, and tryptophane and between these amino acids and the coenzymes reduced diphosphopyridine nucleotide and oxidized riboflavin. The significance of the mechanism of resonance transfer of excitation energy is briefly discussed.

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# *THE DEPENDENCE OF PNEUMOCOCCAL TRANSFORMATION ON THE MOLECULAR WEIGHT OF DEOXYRIBOSE NUCLEIC ACID*

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Increasingly quantitative studies of bacterial transformation[1–3] induced by deoxyribose nucleic acid (DNA) have made this a unique tool for analyzing the genetic function of DNA in terms of its molecular structure. By inflicting known amounts of relatively mild damage on transforming DNA and observing the resultant loss in transforming activity, one may gain an intimate knowledge of the molecular features which are responsible for its genetic function. Thus far, it has been chiefly damage induced by various kinds of radiation that has been studied quantitatively,[4–6] but in these cases it has not been possible to assay directly the chemical and physical damage involved. There is, therefore, a need for means by which single types of damage can be imposed and the extent accurately measured, because only in this way can a direct correlation be made between loss in transforming activity and the fractional amount of damage to a particular feature of the molecular structure of DNA.

As a first approach in this direction, we have sought to find the effect on bacterial transformation of breaking the very long DNA molecules into successively smaller pieces. In this case the amount of damage, if confined only to double chain scission, can be obtained at once from the molecular weight of the degraded samples, and the correlation of this with transforming activity should lead to the minimum length of DNA required for the act of transformation if such a minimum length does exist.

Such an investigation requires the availability of samples of transforming DNA covering a range of molecular weight. This requirement has been met by the studies of Doty, McGill, and Rice[7] on the sonic degradation of DNA. They showed that the exposure of solutions of DNA to 9-kilocycle sound waves causes the fragmentation of DNA by the scission of opposite pairs of phosphoester bonds without disturbance of the remainder of the paired polynucleotide strands. As a result, samples of structurally homologous DNA can be prepared which differ physically only in molecular weight.

We report here the preparation of several DNA samples from pneumococcus, the production of sonicates (degraded samples) covering more than a tenfold range in molecular weight, and the correlation of the transforming activity with the molecular weight.

## EXPERIMENTAL DETAILS

*Bacterial Strains.*—The recipient strain used in the transformation experiments was a variant of rough strain R-36A *Diplococcus pneumoniae* derived originally from Type II. In most experiments, the DNA was isolated from a donor strain which carried five unlinked resistance markers: streptomycin, micrococcin, eryth-

romycin, novobiocin, and optochin. The donor strain was obtained by transforming the recipient strain with respect to each of these characters, one at a time. The micrococcin-resistant strain was obtained from Dr. R. D. Hotchkiss, the erythromycin strain ($ery_2$) from Dr. A. W. Ravin, and the optochin strain from Dr. L. Lerman.

*Isolation of DNA.*—Active transforming DNA was isolated from mass cultures of the donor strain by lysis of the cells with sodium deoxycholate, followed by alternate deproteinizations with chloroform and precipitations of the DNA with ethanol. Ribonucleic acid was removed by overnight incubation in the cold with ribonuclease, followed by dialysis for 24 hours against 2 *M* NaCl. To insure complete removal of the ribonucleic acid, several precipitations were carried out with isopropanol (0.54 volume fraction) in the presence of 0.3 *M* sodium acetate, according to Simmons. The DNA was never exposed to ionic strengths less than 0.01.

*Sonic Degradation.*—Sonic degradations were carried out for various lengths of time (1 minute–3 hours) on small volumes (2–3 cc.) of solutions contained in nitrocellulose centrifuge tubes suspended in water within the cup of a Raytheon magnetostriction sonic oscillator (50-watt).

Molecular weights were derived from sedimentation constants ($s_{20,w}^0$) using the relation established by Doty, McGill, and Rice:[7]

$$s_{20,w}^0 = 0.063\ M_w^{0.37}.$$

The sedimentation constants were determined from the median point on the photometer trace of the photographs taken with the ultraviolet absorption optics in the Spinco Model E ultracentrifuge at concentrations less than 30 mg/l. Intrinsic viscosities extrapolated to zero gradient were determined for two undegraded samples and found to be 33 for TP-32-M and 60 for Ca-Str-15 in units of 100 cc/gm. These values led to the same molecular weights as the sedimentation constants when the corresponding intrinsic viscosity–molecular-weight relation[3] was employed. The probable error associated with the molecular weights is ±15 per cent.

In an initial set of experiments, sonic irradiation was carried out with the DNA dissolved in the standard saline-citrate solvent (0.015 *M* NaCl plus 0.015 *M* sodium citrate). The results are shown in Figure 1 as the logarithm of transforming activity plotted against reciprocal molecular weight. Since it had been shown that denaturation temperature of DNA was not lowered by sonic irradiation,[7] we thought at first that indirect effects were absent. However, it was found that the activity of a sample degraded to a given molecular weight was somewhat dependent on the concentration of the DNA solution irradiated with sonic waves. The addition of 5 per cent of *S*-(2-aminoethyl)isothiuronium bromide hydrogen bromide (AET),[8] kindly supplied by Dr. D. G. Doherty, eliminated the concentration dependence and led to samples having higher activity for a given molecular weight. Some results for some samples produced from the same undegraded DNA in the presence of AET are also plotted in Figure 1. As a consequence, 5 per cent AET was used in the preparation of subsequent sonicates. The AET was removed by dialysis after the sonic treatment. The sedimentation constants and derived weight average molecular weights are listed in Table 1.

*Transformation.*—The samples were assayed for transforming activity, using glycerol-treated recipient cells stored at −15° C.[2] Exposure of the recipient cells

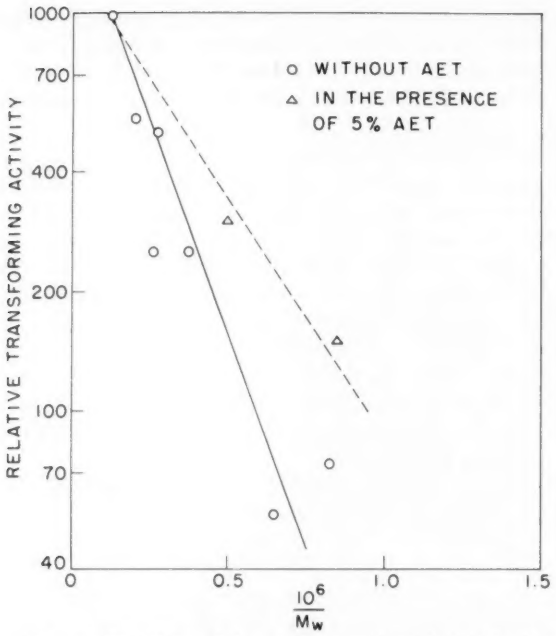


FIG. 1.—Logarithm of the relative streptomycin-transforming activity of sonic fragments prepared in the absence and presence of AET plotted against the reciprocal molecular weight.

TABLE 1

SEDIMENTATION CONSTANTS, MOLECULAR WEIGHTS, AND RELATIVE TRANSFORMING ACTIVITIES OF UNDERGRADED AND SONICATED SAMPLES OF DNA

| Sample | $(s_{20,w})^0$ Median | $M_w \times 10^{-6}$ | Relative Activity |
|---|---|---|---|
| *TP-34-M:* | | | |
| Control | 23 | 8.0 | 1,000 |
| Sonicate | 9.3 | 0.87 | 100 |
| *Ca-Str-15:* | | | |
| Control | 23 | 8.0 | 400 |
| Sonicate (1) | 12.7 | 2.1 | 112 |
| Sonicate (2) | 10.7 | 1.2 | 72 |
| *TP-32-M:* | | | |
| Control | 15.5 | 3.5 | 150 |
| Sonicate (1) | 14.8 | 2.8 | 132 |
| Sonicate (2) | 13.1 | 2.0 | 102 |
| Sonicate (3) | 10.3 | 1.1 | 41 |
| Sonicate (4) | 8.6 | 0.72 | 13 |
| Sonicate (5) | 6.3 | 0.32 | 0.3 |

to DNA at 32° C. was terminated after 30 minutes by the addition of deoxyribonuclease (Worthington, crystalline). The cells were then incubated at 37.5° C. for 90 minutes and scored in appropriate selective media. The DNA concentration used in transformation was always sufficiently low to insure that the number of transformants was proportional to the concentration.[5]

Comparisons of the activity of the sonicates with the undegraded samples were made by measuring the relative number of transformants induced in aliquots of a single recipient culture by equal weight concentrations of the various DNA samples. In nearly all cases the conditions for assay were adjusted so that at least 100 transformed cells would be counted. As a consequence, the limits of error in the assays can be set as ±20 per cent from the 95 per cent confidence limit of the Poisson distribution. The maximum efficiency of transformation, using undegraded samples, was about 1 per cent.

## RESULTS

*Undegraded Samples.*—A number of different samples of DNA have been made from pneumococci. The molecular weights fell within the range of 3–8 million, and the activities (streptomycin transformation), with occasional exceptions, spanned a range of 10. There was a rough correlation between activity and molecular weight, but occasionally a sample of unusually low activity was prepared. Two samples used for the preparation of sonicates had the highest activity and the highest molecular weight of all the samples prepared (Ca-Str-15 and TP-34-M). It is interesting to note that the molecular weight in these cases matched the highest values of molecular weight obtained from calf thymus and other sources.[9, 10] The other sample used (TP-32-M) was typical of the majority of preparations we have made. The highest streptomycin-transforming activity was shown by sample TP-34-M, and it has been assigned a value of 1,000. All other activities are recorded relative to this value.

*Sonicates.*—From the three original DNA samples just mentioned, a total of 8 sonicates was prepared, characterized, and assayed. The logarithm of the relative activity in streptomycin transformation of these 11 samples is plotted in Figure 2 as a function of reciprocal molecular weight. Since substantial evidence has been obtained[11, 12] to show that the molecular-weight distribution of the sonicates is essentially that of the most probable distribution (see below), it follows that the reciprocal molecular weight is directly proportional to the fraction of bonds broken. Consequently, this quantity also serves as an abscissa and is indicated in Figure 2. Simultaneous assays were carried out on most of the samples for transformation to erythromycin and micrococcin resistance. The relative activities were the same within probable error. These results demonstrate, therefore, that, although the undegraded samples differ among themselves, the sonicates of any undegraded sample show the same decay in activity with decreasing molecular weight for three different genetic markers. This result—a direct proportionality between the logarithm of activity and the fraction of bonds broken—is the simplest possible, but its interpretation depends upon an analysis of the transformation process.

*Analysis of First-Order Decay as a Function of Reciprocal Molecular Weight.*—An essential feature of the transformation assays in this work was the use of recipient cells in such a state that transformation in them is induced at a constant rate over the period of exposure to the DNA (30 minutes). Fox and Hotchkiss[2] have pointed out that, under these conditions, the rate of induction of transformation shows a dependence on DNA concentration resembling that of the Michaelis-Menten mechanism of enzyme kinetics. Thus they suggest that a rapidly established equilibrium is set up between free DNA and DNA reversibly bound at a

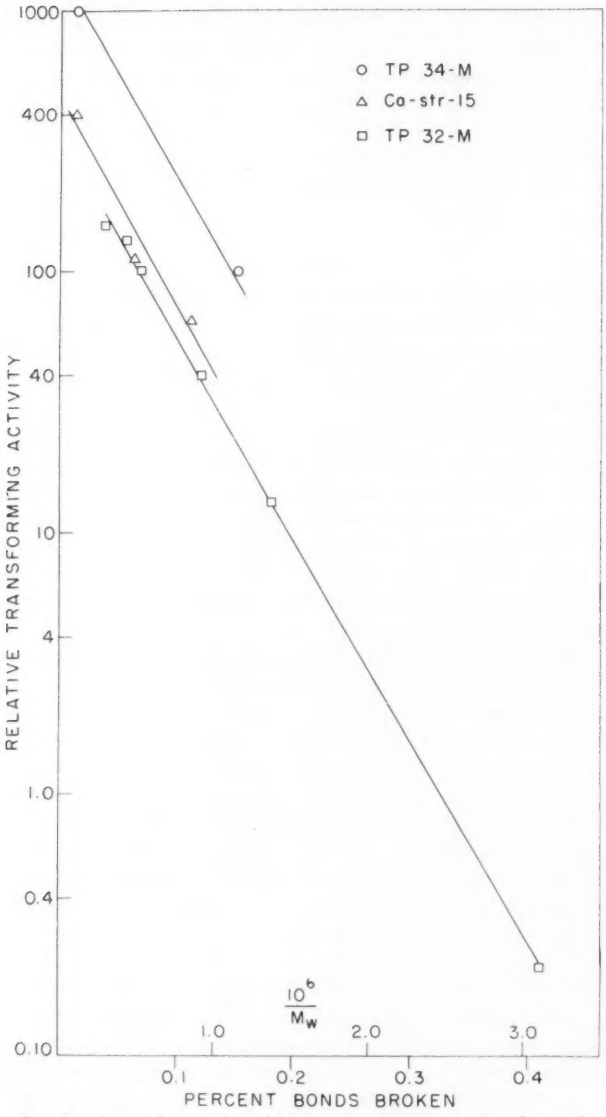


FIG. 2.—Logarithm of the relative streptomycin-transforming activity of sonic fragments prepared in the presence of AET plotted against the reciprocal molecular weight and per cent bonds broken.

bacterial site. Transformation is then proportional to the irreversible incorporation of DNA from the DNA-site complex:

$$\text{DNA} + \text{site} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{DNA-site complex} \xrightarrow{k_3} \text{incorporated DNA} + \text{site}.$$

It is convenient to discuss our results in terms of this mechanism, but it must be kept in mind that the concentration of DNA must now be considered on a molar basis rather than on a weight basis. The latter choice is satisfactory for conventional experiments where the ratio of the weight and molar concentrations is a constant, but it does not hold when the molecular weight is varied, as in our case.

As in enzyme kinetics it is assumed that the concentration of DNA, the analogue of the substrate, is much greater than the concentration of bacterial sites. In this case the velocity of incorporation is given by

$$v_i = \frac{k_3 \text{ site } [\text{DNA}]}{(k_2/k_1) + [\text{DNA}]}, \tag{1}$$

where DNA represents the *molar* concentration of DNA. If we now assume that (1) the same values of the three rate constants apply to all molecular species of DNA, (2) a fraction $z$ of the DNA molecules contains the active markers, and (3) transformation will occur with a probability $p$ when an active marker enters the cell, the rate of transformation, $v_t$, is given by

$$v_t = pzv_i = \frac{pzk_3 \text{ site } [\text{DNA}]}{(k_2/k_1) + [\text{DNA}]}. \tag{2}$$

When the concentration of DNA is sufficiently small, $[\text{DNA}] \ll k_2/k_1$, and

$$v_t = pz \frac{k_3k_1}{k_2} [\text{site}] [\text{DNA}]. \tag{3}$$

This region of low DNA concentration corresponds to the linearly increasing portion of the transformation versus concentration plot, where the assays reported here were performed. When the activities of two samples of DNA are determined, using aliquots of the same culture of recipient cells, it is the ratio of values of the product, $pz(k_3k_1/k_2)$ [DNA], which is obtained. The observed variation of transforming activity with molecular weight may therefore result from a first-order decay in one or more than one component of this product. If, for example, the sonically induced chain scission had only the effect of destroying active markers when a scission occurred within them, the effect would be described as a first-order decay in $z$[DNA], since this product equals the number concentration of markers. Alternatively, if there were a minimum molecular size required to form the DNA-site complex, the molar concentration of DNA would have to be corrected for the amount that did not have the minimum molecular weight. That is, [DNA] would mean the molar concentration of material having molecular weights in excess of the minimum value.

We consider these two possibilities—decay in number of active markers and gradual conversion of DNA into pieces too small to form the DNA-site complex—to be the most likely cause of the observed fall in activity with the fraction of bonds broken in the sonicates. Analyses of these two effects lead to the conclusion that both will display a first-order, logarithmic, decay as a function of the fraction of bonds broken. We summarize here the argument for the case of inactivation due to damage of markers and postpone until later the case involving a minimum length for attachment.

Since the inactivation of a marker is assumed to take place when a double-chain

scission occurs within it, this event may be considered a hit and the conventional target theory applied. In this context, let $H$ be the number of hits per gram of DNA, $N$ the number of markers per gram of DNA, $S$ the number of double-chain scissions per gram of DNA, and $M_c$ the molecular weight of the sequence that constitutes the marker. A fraction $1/e$, or 0.37, of the markers will survive when the number of hits becomes equal to the number of markers. Using the subscript 37 to denote quantities relating to the 37 per cent dose, $H_{37} = N$. For the 37 per cent dose, the fraction of double-chain scissions which occur within active sequences is $NM_c$; the total number of breaks which occur within active sequences is therefore given by $H_{37} = S_{37}\ NM_c$. Equating these two expressions for $H_{37}$ gives $M_c = 1/S_{37}$.

We have reason to believe that all our DNA samples, both degraded and undegraded, have molecular-weight distributions which may be represented approximately by a "most-probable" or Flory distribution.[13] For this distribution, $q$, the fraction of bonds broken, is given by

$$q = 2/x_w = 2M_0/M_w,$$

where $x_w$ is the weight average degree of polymerization, $M_w$ is the weight average molecular weight, and $M_0$ is the molecular weight of a single nucleotide pair. Since $S$ is given by

$$S = qN_0/M_0 = 2N_0/M_w,$$

where $N_0$ is Avogadro's number, a plot of the logarithm of relative activity versus the reciprocal weight average molecular weight should be a straight line, as is indeed observed. For the 37 per cent dose,

$$S_{37} = q_{37}N_0/M_0;$$

therefore,

$$\begin{aligned} M_c &= M_0/q_{37}N_0 \text{ (in grams)} \\ &= M_0/q_{37} \text{ (in molecular-weight units).} \end{aligned}$$

If it is recognized that our starting material was not infinitely long DNA, but had a finite fraction of bonds already broken, this may be denoted by $q_{100}$. This is then used to correct the critical molecular weight:

$$M_c = M_0/(q_{37} - q_{100}) = 660/(q_{37} - q_{100}).$$

Using this result and the slope of the lines in Figure 2, one arrives at the value of 1.0 million for $M_c$.

The alternative analysis, involving the conversion of DNA to pieces too small to form the complex, leads to a result that is formally the same as this one, provided that the molecular-weight distribution remains the most probable one. Consequently, if it is considered that the loss in activity due to sonication occurs only as a result of reduction in molecular size below that required for attachment, one is led to expect that the logarithm of the relative activity will fall linearly with the reciprocal molecular weight and that the observed slope corresponds to a critical molecular weight of 1 million for attachment.

## DISCUSSION

Thus two alternative interpretations of the data are possible. The critical value of the molecular weight determined is the same in each case, but it has two quite different meanings. The fact that it is the same for three different markers suggests that it represents a common requirement for transformation that is independent of the markers themselves, which, a priori, would be expected to be of smaller and variable molecular weights. This argues that the critical size refers to the attachment size. On the other hand, the active marker may be large enough to have been significantly damaged by the chain scissions. A discussion of this possibility requires a clarification of the term active marker used above. Our use of this term in connection with equation (2) implies that this is the minimum length of DNA required for transformation after passage through the attachment site. Its minimum size would presumably refer to the minimum length of DNA required to contain the nucleotides that are altered in the mutation that gives rise to the altered strain. From the work of Benzer it appears that this *may* be as small as a few nucleotides. However, this minimum size, which may be called a mutational site, could be considerably smaller than the active site. For example it may have to be located well away from the ends of a DNA molecule in order to participate in recombination or the act of recombination itself may have a minimum size requirement. Thus although the mutational site or genetic marker itself may well be so small that it escapes damage during the sonication, the region required for its expression after incorporation may be large enough to contribute to the critical size measured in these experiments. The simultaneous operation of both of these effects would still lead to the observed behavior. We are therefore, at present, undertaking experiments designed to resolve the relative contribution of each of these processes to the observed decay in activity.

It follows from these considerations that the critical molecular weight of 1.0 million obtained here refers to the minimum molecular weight for attachment or for incorporation into the genetic material of the cell or to the combined effect of these two limiting processes. It is clear, therefore, that the critical molecular weight found here does not bear any direct relation to the radiation sensitive volume obtained from radiation inactivation experiments[4-6] and should not be compared directly with these other results.

## SUMMARY

Exposure of solutions of transforming factor to 9-kilocycle sound waves causes scissions of the molecules with a concomitant fall in transforming activity. The addition of *S*-(2-aminoethyl)isothiuronium bromide hydrogen bromide during sonic treatment eliminated inactivation of transforming factor by secondary effects. By plotting the logarithm of the relative transforming activity of the sonic fragments versus the reciprocal molecular weight and analyzing the curves by the conventional target theory, the critical molecular weight for streptomycin, erythromycin, and micrococcin was found to be 1.0 million. The value may reflect either the minimum size required for effective attachment to a bacterial site or a combination of this and the minimum size required for genetic incorporation following entrance into the bacterial cell.

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$$W_x = xq^2(1 - q)^{x-1},$$

where $q$ is the probability of a bond having been broken.

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## *OBSERVATIONS IN MYELIN STRUCTURE: INCISURES AND NODAL REGIONS**

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Biophysical studies employing polarization optics, X-ray diffraction, and electron microscopy have provided valuable information concerning the layered structure of nerve myelin at the molecular level.[1–4] Electron microscope studies of the myelination process by Geren[5] led to the concept that the lipid-protein layered structure, which repeats very regularly in the radial direction, is produced by the wrapping about the axon of many folds of the surface membrane of the Schwann cells, which come to surround the outgrowing axon. Geren's findings have been confirmed by a number of investigators (see particularly Robertson[6]), so that the general concept has now been well established. However, the structure of the myelin at the Schmidt-Lanterman incisures and at the nodes of Ranvier remains to be determined, and it is with this problem that the author's investigation deals. A

full understanding of the structural relations at incisures and at the nodes would be of significance not only in evaluating the Geren hypothesis but also because of its bearing on physiological processes, such as impulse propagation. In the present paper we shall be concerned primarily with the structure of the incisures. The nodal structure has also been dealt with,[7, 8] but the results obtained at that time were not definitive. In more recent work Robertson[9–11] has furnished important new evidence on nodal structure.

*Methods and Materials.*—The peroneal and cubital branches of frog sciatic nerves were used in all experiments. Fixation was in Palade's fixative made isotonic with frog blood, pH 7.4, and containing potassium ions (6 mM) and calcium ions (4.2 mM). After imbedding in methacrylate, thin sections were cut with a modified Minot microtome. Glass knives were usually used, but in some cases a diamond knife, kindly provided by Dr. H. Fernandez-Moran, was employed. The observations were made with an RCA type EMU-2B electron microscope provided with a compensated pole piece and a 50-$\mu$ aperture.

*Results.*—Incisures are readily recognized in thin transverse sections of myelinated fibers because at these levels the myelin appears as two concentric ribbons separated by a relatively empty space (Fig. 1). Mitochondria and other cytoplasmic organelles, including granules of the type described by Gasser,[12] were not observed in this space. However, in fortunate sections it is seen that the lumen of the incisure is traversed by thin ribbons which may be seen to be continuous with the spiral wrapping of the myelin on either side of the incisure. Within the incisure the ribbons disappear from sight because of the thinness (*ca.* 200–400 A) of the section.

To test the idea that the layers do in fact traverse the incisures, longitudinal sections were studied. Only when traction was applied to the fiber, did the incisures appear as distinct clefts or channels. The surface membrane of the axon (axolemma) was continuous under the incisure; hence there is no continuity between axoplasm and incisural space.

In fibers which had been protected as much as possible from traction, the continuity of the myelin layers across the cleft was manifest. In Figure 2, which is fairly typical of such preparations, it may be seen that layers peel off tangentially and, sometimes after changing direction several times, are incorporated into the layered structure on the other side of the cleft.

Thus it is seen that the so-called incisures do not constitute channels[12–14] through which materials may be exchanged between the axon interior and its environment (see Stoeckenius and Zeiger[15]) or through which ions may flow (unless this could occur in the extremely thin space, unresolved in these electron micrographs, between pairs of apposed membranes). Rather, the incisural space is traversed by as many membranes as occur in the compact myelin. The resistance to diffusion or ion flow is therefore similar in both regions.

This structure is consistent with Geren's membrane theory of myelin formation. With rotation of Schwann cell with respect to axon, Schwann cell protoplasm is squeezed out from between the membrane pairs. If protoplasm is squeezed out in the axial direction, as the spiral is tightened and layer condensation occurs, protoplasmic constituents might be expected to accumulate in the regions of the incisures and at the nodes.

If incisures are not diffusion pathways, one may perhaps venture the suggestion

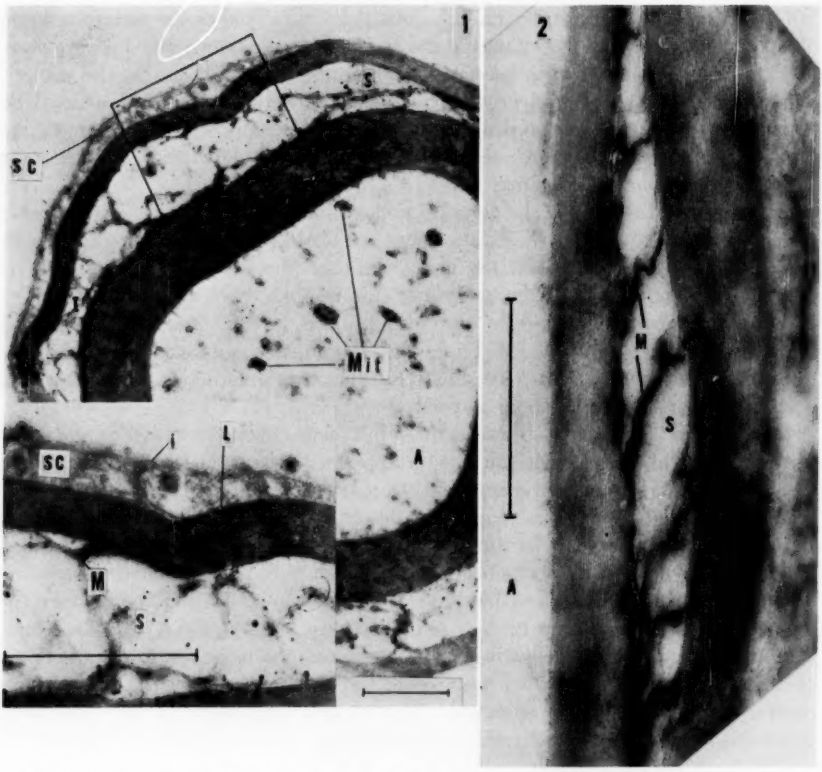


FIG. 1.—Transverse section of a myelinated nerve fiber at the level of an incisure. *SC*, Schwann cell; *S*, incisural space; *i*, infolding of Schwann cell surface membrane which fuses with compact myelin at *L*; *M*, myelin layer projecting into incisural space; *Mit*, mitochondria; *A*, axoplasm. Magnification, 22,000. Blocked-out region shown in insert at higher magnification (51,000).

FIG. 2.—Longitudinal section at the level of an incisure. The continuity of myelin layers on both sides of the incisure is clearly shown. Legend as before. Magnification 56,000.

that, somewhat along the lines suggested by Glees,[16] they may play a role in mechanical adaptation to distorting influences by a kind of telescopic distension of the cylindroconic internodal segments.

Another interesting feature of myelin structure, which is also clearly consistent with Geren's membrane hypothesis, is the peeling-off of the myelin layers near the node, with a change of direction varying from 45° to as much as 135°, to reach the axolemma upon which they are inserted. In Figure 3 this peeling-off of layers is clearly shown, although the axolemma is not evident. Between the detached layers which have already changed direction, empty spaces occur; these had already been observed by histologists and may well correspond to the "spiny bracelets" of Nageotte (see Gasser[12]).

In the nodal region one may observe the ending of double layers of the myelin. It will be recalled that in the Geren model the dark lines represent two primitive membranes which have fused. As the fusion of spiral wrappings proceeds, Schwann

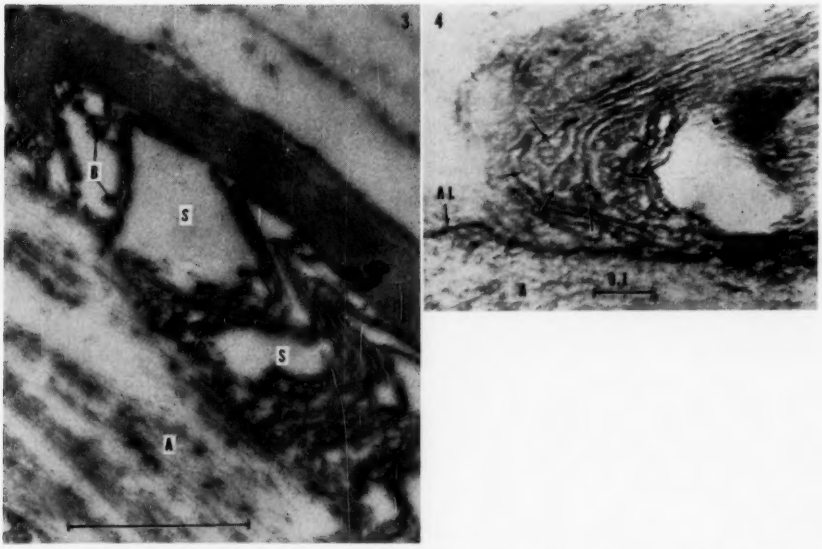


FIG. 3.—Longitudinal section showing termination of myelin layers near the node of Ranvier. *B*, loop profiles at end of myelin layers; other lengends as before. Magnification 56,000.

FIG. 4.—Pear-shaped profiles of endings of myelin layers near node of Ranvier depicted with arrows. *AL*, axolemma; other legends as before. Magnification 180,000.

cell protoplasm is squeezed tangentially and axially, accumulating in cylindrical sacks at the nodal end of each membrane doublet. Loops or pear-shaped endings, *ca.* 300 A in diameter and corresponding to the profiles of such sacks, were seen very frequently in our sections. Examples are shown in Figure 4. In other cases the loops have opened up, suggesting that, because of pressure generated during development, the sacklike endings may have ruptured.

*Summary.*—The layered structure of the myelin sheath of frog nerves is discussed, with particular reference to the region of the Schmidt-Lantermann incisures. It is shown, in the incisure, that the membrane doublets peel off from one side of the compact myelin, traverse the open space, and join the compact myelin on the other side. The incisure is thus not a diffusion channel from axolemma to extracellular space and is unlikely to be involved in current flow during impulse propagation. The structure of the myelin near the node is also briefly described. These structures are consistent with Geren's membrane theory of the origin of nerve myelin.

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## *THE INACTIVATION OF GROWTH-PROMOTING DISLOCATIONS WITH TEMPERATURE, PRESSURE, AND POISONS**

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### INTRODUCTION

Crystal growth involves catalytic structures usually lumped together as "nucleation." Such structures may be screw dislocations or some other bridge between phases. In any case this bridge structure will have thermodynamic properties different from those of the crystal in bulk. Such catalytic structures are, in particular, to be expected for the growth of ice crystals on crystals of silver iodide. In our theory we assume that there are $\delta$ possible sites per square centimeter, of which $\beta$ are active as catalysts and the number $\gamma$ are deactivated. We suppose further that $\beta$ and $\gamma$ are in dynamic equilibrium.

### THEORY

The data to be explained are given as delay time for ice formation in a supercooled liquid-vapor mixture, in which vapor is therefore supersaturated with respect to ice, seeded with a standard amount of silver iodide crystals. The reciprocal of this delay time should be proportional to the velocity of ice formation.

At low temperatures the apparent activation energy is 13.8 kcal. This represents the heat of vaporization of supercooled water droplets plus a small activation energy. At high temperatures, above $-17°$ C., one obtains an apparent activation energy of $-40.4$ kcal. In this case we suggest that a rapidly established equilibrium exists between surface active sites (dislocations) at which ice formation can

occur and inactive sites, with the total number of sites remaining constant.

This may be represented as follows:

$$\begin{array}{ccccc} & \alpha & & \beta & k_1 \\ H_2O + & \text{Active site} & & \rightleftarrows & \text{Ice.} \\ & \Downarrow\Uparrow & & & k_2 \\ & \text{Inactive site} & & & \\ & \gamma & & & \end{array}$$

Now the velocity of ice formation, $v$, is given by

$$v = \alpha\beta k_1 - \beta k_2, \tag{1}$$

and we may write, for the total number of sites,

$$\begin{array}{ccccccc} \text{Total sites} & = & \text{Active sites} & + & \text{Inactive sites} \\ \delta & = & \beta & + & \gamma \end{array}$$

or

$$\frac{\gamma}{\beta} = K, \qquad \beta = \frac{\delta}{1 + K}. \tag{2}$$

Now we note that

$$v = \frac{\delta}{1 + K}(\alpha k_1 - k_2), \tag{3}$$

and, if $\alpha k_1 \gg k_2$ at low temperatures,

$$v = \frac{\delta}{1 + K}\alpha k_1 = \frac{AT\, e^{-\Delta H\ddagger/RT}}{1 + e^{-\Delta H/RT} e^{\Delta S/R}}. \tag{4}$$

Theoretically, $k_1$ is equal to the specific rate for passing over the highest barrier along the reaction path divided by the total number of barriers lying within the energy range, $kT$, below this highest barrier.[1]

In equation (4) we have introduced the absolute-rate theory for $k_1$ and expressed the equilibrium constant, $K$, in terms of $\Delta H$ and $\Delta S$. It is also convenient to include the temperature-independent terms, $\mathfrak{K}k/h$ and $e^{\Delta S\ddagger/R}$ in the constant $A$. Equation (4) has been previously used to account for the observed phenomena in certain bioluminescence experiments.[2] One may show that the condition for a maximum in the velocity given by equation (4) is

$$1 + \frac{\Delta H\ddagger}{RT} - \frac{e^{-\Delta H/RT} e^{\Delta S/R}}{1 + e^{-\Delta H/RT} e^{\Delta S/R}} \left(\frac{\Delta H}{RT}\right) = 0. \tag{5}$$

From the experimental data it is found that the maximum velocity (minimum time delay) occurs at about $-17°$ C. Using the values $\Delta H = 54.2$ kcal. and $\Delta H\ddagger = 13.8$ kcal., one obtains $\Delta S = 210$ e.u. This is quite close to the values found for certain biological reactions where it is known that relatively large molecules are involved and the process is the breaking of a number of hydrogen bonds.

Figure 3 presents a comparison of observed and calculated values of the delay time for ice formation and was calculated from equation (4).

It is interesting to consider the effect of changing external pressure on the rate of growth of ice clouds in a condensed system. This could be done by growth in water or in a solvent in which water has limited solubility and which could be subjected to external pressure. In this case equation (5) should be written thus:

$$v = \frac{ATe^{-\Delta H\ddagger/RT}e^{-(p-1)\Delta v\ddagger/RT}}{1 + e^{-\Delta H/RT}e^{\Delta S/R}e^{-(p-1)\Delta v/RT}}, \quad (6)$$

in which $p$ is the external pressure in atmospheres (note that the rate and equilibrium constants are usually measured at one atmosphere) and $\Delta v$ is the volume per mole of the final state less that of the initial state, while $\Delta v\ddagger$ is the change in volume per mole of the activated complex less that of the initial state.

Foreign molecules may be expected to lessen the stability of "growth-promoting dislocations" in two possible ways, paralleling the two ways in which poisons inhibit enzymes. The first type of inhibition of growth arises when a foreign atom or molecule occupies a position on the dislocation to the exclusion of a molecule needed for the growth process. Thus sulfanilamide poisons luciferase in this fashion. In the second type of inhibition, the inhibiting molecules shift the equilibrium state of the growth-promoting dislocation toward the inactive form. Three alcohol molecules inhibit luciferase by "denaturing" it in this latter manner.[2] A study of enzyme denaturation, as reported by Johnson *et al.*,[2] is extremely suggestive for further studies of growth-promoting dislocations.

## EXPERIMENTAL

*Description of Apparatus.*—The mixing cloud chamber used in this work is shown in Figure 1. Cooling was by mechanical refrigeration. The nitrogen stream entering the chamber was at 25° C. and 96 per cent relative humidity, with a flow rate of 5 liters/minute. The cloud-chamber temperature was indicated by two "aniline-point" mercury-in-glass thermometers, supported free of the wall. The lower thermometer was 1 mm. from the bottom of the chamber, and the upper thermometer was 20 cm. from the bottom. The upper thermometer usually indicated 1° or 2° higher than the lower, though this difference increased to 7° at the lowest temperature used. In all cases, the mean of these readings was used as the run temperature. The source of AgI aerosol used to cause transformation of the supercooled water cloud to ice is not shown, having been described previously.[3] It consisted essentially of a Vycor trap held at 600° C., with 7 gm. of AgI in the bottom and dry $N_2$ flowing into the side arm at 500 cc/minute. The effluent from the center tube was passed through a dry ice trap to remove $I_2$ vapor, and the remaining output, a blue scattering aerosol plainly visible in a light beam, was used as the nucleating material. A 50-cc. hypodermic syringe with a No. 15 needle and a 1-liter three-necked flask comprised the aerosol dilution and handling apparatus.

*Experimental Procedure.*—The nitrogen streaming into the cloud chamber at 5 liters/minute caused considerable turbulence. This was considered desirable, since thereby each parcel of the cloud could be expected to experience a sample of the conditions throughout the cloud chamber. For this reason the sample of nucleating aerosol was always injected as strongly as possible, to provide an additional stirring action and to mix the aerosol and cloud as thoroughly as possible.

The direct AgI aerosol output after trapping the $I_2$ was chosen as unity relative

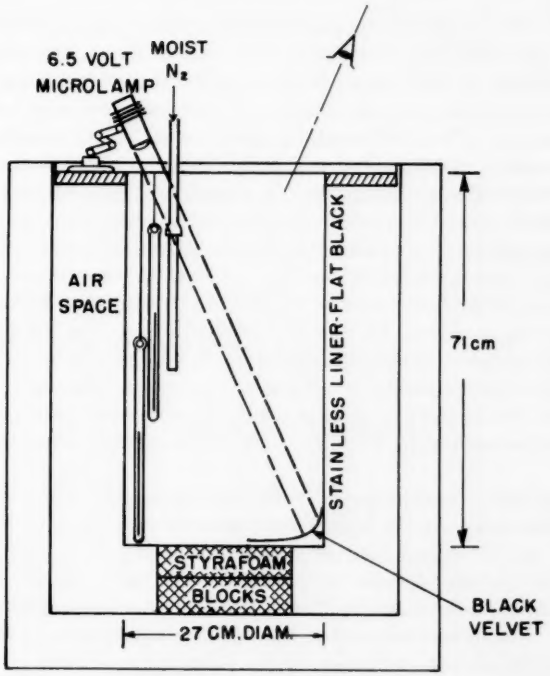


FIG. 1.—Mixing cloud chamber.

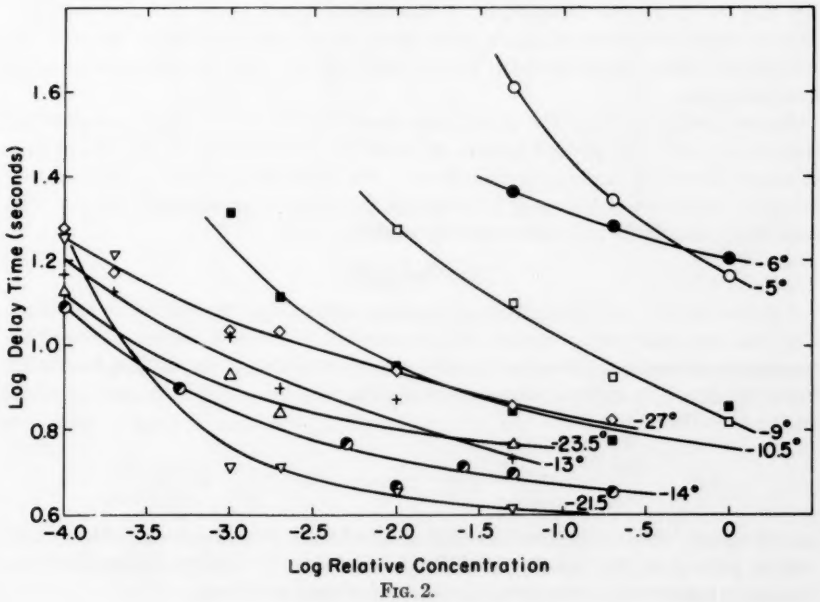


FIG. 2.

concentration. This direct output was run into the temporary storage flask (1-liter, three-necked) directly for unity concentration tests. The flask was then carried to the cloud chamber, and a 50-cc. sample was withdrawn with the syringe and immediately injected into the cloud. The time delay between this injection and the appearance of brightly scintillating ice crystals was recorded. Tests at concentrations below unity were carried out in a similar manner, the flask having been flushed with clean laboratory air. A sample of AgI aerosol from the direct output was taken with the syringe. This was then either directly added to the temporary storage flask or reduced further in concentration by pumping the syringe in laboratory air prior to injection into the flask. In this way the relative concentration of aerosol in the flask could be prepared to be any value between unity and 0.0001. The tests at various relative concentrations as well as the runs at various cloud-chamber temperatures were randomized in time sequence. This was done to avoid the possible systematic errors due to interaction between successive tests and slow variations in the nuclei output. Blank runs were made occasionally to insure that the aerosol handling equipment was thoroughly cleaned by the flushing procedure.

Several qualitative observations not directly related to nucleation delay time should be mentioned. At the higher temperatures (around $-6°$ C.) a relative concentration of 0.05 caused just perceptible nucleation, with very few scintillations, and short lifetime of these ice particles. The same relative concentration at $-13°$ C. caused an "overseeding," the entire chamber being filled with ice crystals, and over $^1/_2$ hour was required to reduce the concentration of ice crystals to the background level. On reduction of the temperature to below $-20°$ C., the same relative concentration caused similar complete conversion of the cloud to ice, but this ice cloud was replaced by a supercooled water cloud in a few minutes. The threshold temperature above which these nuclei were not active was $-5°$ C. Several tests were run with the $I_2$ vapor trap removed, and no difference in delay time was noted.

*Experimental Results.*—The results are plotted in Figure 2. This presents the time delay in seconds plotted against the relative concentration for a series of runs at temperatures as marked on the curves. By plotting a constant concentration line for relative concentration of 0.05 on rate theory axes, we obtain Figure 3. The solid line in this figure is derived from equation (4).

## CONCLUSIONS

Figure 3 shows that the agreement between theory and experiment is excellent. The heat and entropy for bridge inactivation fall well inside the range found for enzyme denaturation. Enzyme denaturations are likewise responsible for rather sharp temperature optima for many biological reactions. Optima such as these arising from the factor

$$\frac{A_0}{1 + e^{-\Delta F/RT}}$$

can be called "catalyst optima" as distinguished from simple optima, which would still be present in the absence of this catalyst factor. Catalyst optima will undoubtedly prove to be a very general feature of phase transitions.

In the growth of ice crystals on crystalline silver iodide, the time which must elapse before the ice crystals can be observed is a sum of a time, $t_1$, for the first few layers to get started on the silver iodide plus the time, $t_2$, for the remaining million layers to be laid down so that the crystal becomes visible. In the absence of silver iodide, the nucleation time is long compared with the time $t_2$. Successful nucleating agents make $t_1 \ll t_2$, so that one expects to find the same total time, $(t_1 + t_2) \doteq t_2$, independent of which successful nucleating agent is used. We assume that silver iodide is a successful nucleating agent in this sense. The test of this hypothesis is already under way by making use of other nucleating agents.

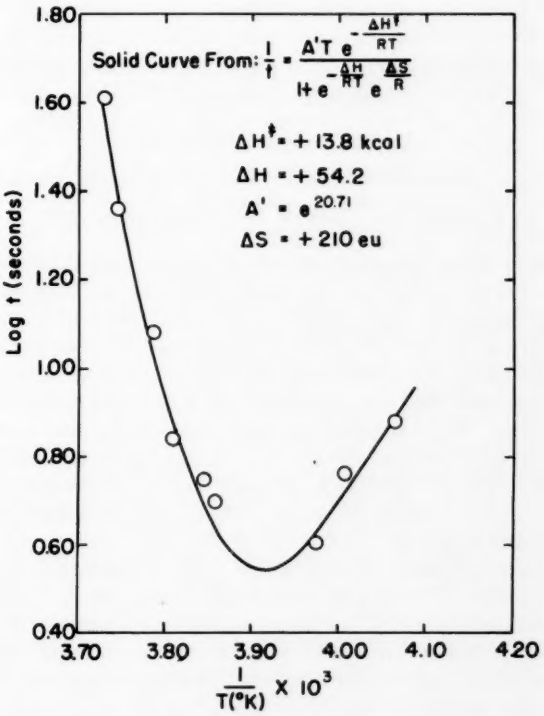


FIG. 3.—Log delay time versus reciprocal temperature.

The number of water molecules in a catalytic unit is calculable, since the catalyst must be composed of metastable ice only slightly less stable than ice in bulk. Thus the number of water molecules, $n$, in the catalytic unit is

$$n = \frac{\Delta H_{n\mathrm{H_2O}}}{\Delta H_{\mathrm{H_2O}}} = \frac{54.2}{1.50} = 36.1 \qquad \text{or} \qquad n = \frac{\Delta S_{n\mathrm{H_2O}}}{\Delta S_{\mathrm{H_2O}}} = \frac{210}{5.42} = 38.7.$$

Both estimates of $n$ should be low by a few per cent, since the ice in the catalytic nuclei is slightly less stable than bulk ice. Thus we can say that very close to 40 molecules must arrange themselves in an appropriate structure to form the cata-

lytic unit. Establishment of the detailed geometrical arrangement of the catalyst structure will require further work.

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## *ON THE ENZYMIC ACTIVITY OF SUBTILISIN-MODIFIED RIBONUCLEASE**

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The initial stage of the proteolysis of bovine pancreatic ribonuclease (RNase) by subtilisin results in the production of a modified RNase molecule whose enzymic activity is very similar to that of the native material.[1, 2] The presence of this altered form of the enzyme (RNase-S) is easily demonstrated by its chromatographic behavior on the Amberlite resin IRC-50, on which it moves as a discrete peak clearly separated from and following the major component (RNase-A) of the native enzyme. The finding of two *N*-terminal amino acid residues and the separation of two peptides from the performic acid-oxidized material indicate that the single peptide chain of RNase-A has been cleaved at one bond and that the two parts remain associated in RNase-S. These original observations have now been confirmed and extended.

Subtilisin appears to attack first the alanyl-seryl bond between residues Nos. 20 and 21, counting from the *N*-terminal end of the RNase-A chain. By careful treatment with trichloroacetic acid it has been possible to separate this *N*-terminal peptide (RNase-S-Pep) from the rest of the molecule (RNase-S-Prot). These fractions have only traces of residual enzymic activity. On mixing a solution of the trichloroacetic acid precipitate with an equivalent amount of the supernatant fluid, the full enzymic activity of the unfractionated material is regenerated.

### EXPERIMENTAL

*Preparation of RNase-S.*—RNase-A was isolated from crystalline ribonuclease, Armour Lot 381-059, by chromatography[3] on IRC-50, dialyzed to remove the phosphate buffer, and lyophilized. This material was treated with subtilisin at pH 8, as described previously,[2] except that the temperature was lowered to 7° C. At this temperature, it was possible to obtain 80–90 per cent conversion of RNase-A to RNase-S without significant further proteolysis. The uptake of alkali, as measured on the pH-Stat,[4] was about 0.9 moles $OH^-$/mole of protein. An end-group deter-

mination on the total digest by the fluorodinitrobenzene procedure revealed bis-DNP-lysine and DNP-serine; other DNP-amino acids were present in insignificant amount. The ninhydrin color of the mixture increased about 10 per cent during the digestion, while no change occurred in the enzymic activity. The total digest was chromatographed on IRC-50 under conditions identical to those used above in the preparation of RNase-A. A trace of material showing enzymic activity was found at the RNase-A position. The RNase-S was eluted more slowly and was separated from the faster-moving material. The bulk of the salt was removed by dialysis for 2 days against distilled water. For final desalting, the solution was run over a mixed-bed resin of IR-120 and IRA-400 in the $H^+$ and $OH^-$ forms, respectively. The eluate was lyophilized and used for the experiments described below.

*Site of Subtilisin Attack.*—RNase-S was oxidized with performic acid, and the products were separated by electrophoresis on glass paper at pH 6 in pyridine-acetate buffer. The eluate of the faster-moving material was hydrolyzed for 24 hours in constant boiling HCl and analyzed by column chromatography,[5] using the sulfonic acid resin XE-69. The ratios of the amino acids obtained closely approximated the following: $Asp_1(MetSO_2)_1Thr_2Ser_3Glu_3Ala_5Phe_1Lys_2His_1Arg_1$. This peptide is characterized by a high content of alanine and the absence of cysteic acid. The composition corresponds exactly[6, 7, 8] to the *N*-terminal 20 amino acids of RNase-A. The presence of a new *N*-terminal serine residue in the protein part of RNase-S also agrees with the known sequence of RNase-A. Thus RNase-S-Pep is composed of the *N*-terminal 20 amino acids of the original RNase-A, whereas RNase-S-Prot accounts for the other 104 amino acids, within the accuracy of the available data.

*Enzyme Assay Procedures.*—Ribonuclease activity employing ribonucleic acid as a substrate was measured either by the Kunitz spectrophotometric procedure[9] or by the acid-soluble nucleotide method, as described by Anfinsen *et al.*[10] A few measurements were made using uridine-2′,3′-phosphate as substrate, also as a spectrophotometric procedure.[11] The accuracy of any of these methods as employed here is no better than ±10 per cent of the measured activity.

*Trichloroacetic Acid Fractionation of RNase-S.*—A solution was prepared containing 1 mg/ml of RNase-S and 4 per cent *w/v* of trichloroacetic acid at 0° C. No precipitate formed at this temperature. A precipitate appeared when the solution was allowed to warm gradually to 30° C. After an additional hour, the precipitate was removed by centrifugation. The supernatant fluid, after continuous extraction with ether to remove the trichloroacetic acid, contained principally RNase-S-Pep. The precipitated protein (RNase-S-Prot) was dissolved, reprecipitated with trichloroacetic acid as before, redissolved, and made up to the original solution volume.

*Regeneration of Enzymic Activity.*—When assayed separately, both the RNase-S-Pep and the RNase-S-Prot solutions showed less than 5 per cent of the activity of unfractionated RNase-S. When equal volumes of the two were mixed, 100 per cent of the original activity was found. No lag was observed in the recovery of this activity, preincubation of the mixture being unnecessary. A lag period of 1 minute could easily have been observed when the Kunitz assay procedure was used. The recovery of activity was essentially instantaneous and complete even in the very dilute conditions of the assay procedures, where both the protein and the peptide components were present at concentrations of less than $1 \times 10^{-6}$ *M*. The very strong interaction between the peptide and protein is emphasized by the data (cf.

Fig. 1) obtained upon the addition of increasing amounts of RNase-S-Pep to a constant amount of RNase-S-Prot. The shape of the curve resembles that obtained in studies[12] on the recombination of the flavin and protein parts of the old yellow enzyme.

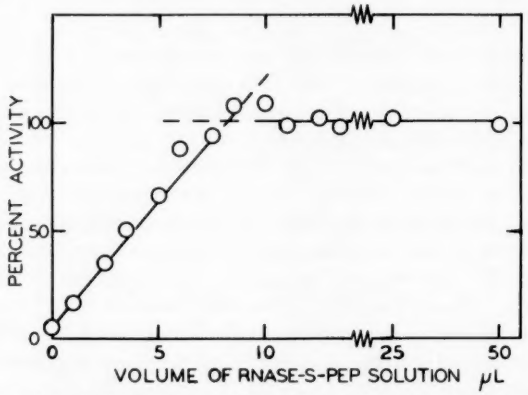


FIG. 1.—Regeneration of ribonuclease activity. The activity of 10-μl. aliquots of RNase-S-Prot solution is shown as a function of the volume of added RNase-S-Pep, both solutions at a concentration equivalent to RNase-S at 1 mg/ml. The maximum regenerated activity thus corresponds to that of 10 μg. of RNase-S. The assays were carried out by the acid-soluble nucleotide method, using 1 ml. of 0.5 per cent ribonucleic acid in 0.1 *M* acetate buffer pH 5.0 as substrate. The solutions to be assayed were added in the volumes indicated. The mixtures were incubated at 25° C. for 15 minutes. The measured activities are expressed as a percentage of that obtained with 10 μg. of RNase-S.

Not only was the peptide obtained by trichloroacetic acid fractionation effective in the regeneration of enzymic activity, but the peptide separated electrophoretically from performic acid-oxidized RNase-S also produced 100 per cent of the expected activity when recombined with unoxidized RNase-S-Prot. The only difference between the latter peptide and the RNase-S-Pep is the conversion of the single methionine residue to the corresponding sulfone. It may be concluded, therefore, that the oxidation state of the sulfur of this methionine residue is not important for the enzymic activity, as estimated by the assay procedures used.

*Effect of Trypsin on the Protein and Peptide Components.*—If a solution of RNase-S-Prot (equivalent to a concentration of RNase-S of 1 mg/ml) is treated at pH 7.5 and 27° C. with crystalline trypsin (10 μg/ml), the ability to regenerate ribonuclease activity, upon mixing with RNase-S-Pep, is completely lost within a few hours. Treatment of RNase-S-Pep with trypsin (under similar experimental conditions) abolishes the regeneration of activity on mixing with RNase-S-Prot. The activity of native RNase is unaffected even after several days of incubation with trypsin under the same conditions. When examined by electrophoresis on paper, a trypsin digest of RNase-S-Pep shows three components, as would be expected from the presence of two bonds known to be sensitive to trypsin in this peptide.[13] The extent of the action of trypsin on RNase-S-Prot is unknown at this time. Neither of these inactive trypsin-digested samples caused any detectable inhibition when added to an assay mixture of undigested RNase-S-Pep and RNase-S-Prot.

*Effect of Carboxypeptidase on the Peptide Component.*—A solution of RNase-S-Pep

(derived from RNase-S at 1 mg/ml) was incubated for several days at pH 8 and 27° C. with carboxypeptidase (40 μg/ml). No significant change was observed in the ability of this solution to regenerate ribonuclease activity upon the addition of the protein component. During carboxypeptidase treatment, however, the ninhydrin color did increase, and the presence of free amino acids was demonstrated by reaction with fluorodinitrobenzene.

*Effect of Periodate on the Protein and Peptide Components.*—A solution of RNase-S-Prot (derived from RNase-S at 1 mg/ml), adjusted to pH 8, and made 0.01 *M* with respect to sodium periodate, was slowly inactivated over a period of 16 hours at 27° C. Native RNase and RNase-S-Pep were largely unaffected by this treatment. An interpretation of this result must await a clear demonstration of the nature of the periodate action. Because of the long time interval involved, residues other than the *N*-terminal serine may be involved.[14]

*Effect of Photo-oxidation.*—Solutions of native RNase, RNase-S-Pep, and RNase-S-Prot in phosphate buffer (pH 8) containing 0.005 per cent methylene blue were shaken in a 37° C. bath and illuminated for 1 hour with a 100-watt bulb at a distance of 1 foot, conditions approximating those described by Weil and Seibles.[15] All three solutions were rendered inactive by this treatment. Furthermore, the inactive RNase-S fractions caused no inhibition of the activity of the untreated control solutions. To the extent that the data of Weil and Seibles may be applied to this experiment, it is tempting to speculate that at least two histidine residues may be required for ribonuclease activity, one of which is the histidine residue occurring in the RNase-S-Pep preparation. The only other residue in this peptide presumably susceptible to photo-oxidation is methionine, and the oxidation of this residue has already been shown to be without effect on the potential activity of the peptide.

*Effect of Concentrated Urea.*—RNase-S was dialyzed for 4 days at 25° C. against 8 *M* urea at neutral pH. The dialyzate showed no enzyme activity by itself, but, upon addition to an RNase-S-Prot solution, ribonuclease activity was regenerated. The peptide thus appeared to be dissociated from the protein by 8 *M* urea. The residual protein, freed from urea by further dialysis against water, showed very little activity, and none appeared upon the addition of RNase-S-Pep. Considerable denaturation of the RNase-S-Prot had evidently occurred. The activity of native RNase is unaffected by similar treatment. If assays are carried out in the presence of 8 *M* urea, no regeneration of activity is seen on mixing RNase-S-Pep and RNase-S-Prot. In agreement with earlier data,[16–18] control experiments showed RNase to be active under these conditions, suggesting that the presence of peptide bond No. 20 in the native enzyme prevents the disrupting effect of urea.

*Residual Activity of the Protein and Peptide Fractions.*—The slight enzymic activity of the RNase-S-Pep fraction may be attributed to the presence of a small amount of RNase-S-Prot, which is slightly soluble in 4 per cent trichloroacetic acid. Successive precipitations of the protein fraction leave similar amounts of activity in the supernatant fluid each time (when assayed in the presence of added RNase-S-Pep). The residual activity (about 5 per cent) of the protein precipitate, however, is not affected by reprecipitation, even though no appreciable amounts of peptide can be demonstrated in supernatant fluids other than the first. This residual activity survives the trypsin digestion described above and is present when assays are performed in 8 *M* urea. It is uncertain whether this activity is caused by a small

contamination with RNase-A, which was not separated during the chromatography on IRC-50, or whether it is an intrinsic property of the RNase-S-Prot molecule.

## DISCUSSION

A distinction is usually made between enzymes composed solely of amino acid residues and those that contain a non-amino acid prosthetic group. The separation of the RNase-S system into an inactive protein and a dialyzable, highly specific "cofactor" suggests that this distinction may not be so sharp as supposed. This finding makes it possible to study the enzymic activity of ribonuclease in a manner similar to that successfully applied in the case of the conjugated catalytic proteins. In particular, it will be of interest to examine the possibility that the peptide fragment of ribonuclease contains the active site and that the remainder of the protein serves the function of an "apoenzyme."

## SUMMARY

As a result of proteolysis by subtilisin, it has been possible to remove a peptide from the *N*-terminal end of the ribonuclease molecule. This peptide and the residual protein separately have less than 5 per cent of the original activity of the enzyme. Under certain conditions recombination of the two parts results in full restoration of the activity. The effect of various enzymic and chemical modifications of the two parts on this restoration of activity is described.

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# *ON THE MECHANISM OF SQUALENE BIOGENESIS**

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The discovery that the branched carbon compound mevalonic acid[1] (MVA) is a potent precursor of squalene and cholesterol[2] has been an important advance in the understanding of polyisoprene biosynthesis. Subsequent isotopic studies have shown that the carboxyl group of MVA is lost on the way to squalene[3] and that the remaining 5-carbon fragment is preserved in the terpene chain without rearrangement of the carbon skeleton.[4,5] It is therefore valid to assume that the MVA-derived condensing units are linked by interaction of $C_5$ of one molecule with $C_2$ of another (Fig. 1). In the present communication we wish to propose a general mechanism for the conversion of mevalonic acid to squalene. For reasons which will become apparent, squalene synthesis from branched-chain subunits will be treated as a two-stage process, namely, (*a*) the condensation of three "isoprenoid" units to a sesquiterpene ($C_{15}$) and (*b*) the reductive coupling of two molecules of the $C_{15}$ intermediate to squalene.

The known mechanisms for the elongation of carbon chains in biological systems are condensations of the aldol-, acyloin-, or Claissen type. In all cases, at least one of the reactants has a carbonyl function and the condensation products are ketones, keto-alcohols, or glycols. On the other hand, neither $C_2$ nor $C_5$, the two reacting carbon atoms of MVA, has a carbonyl function, a fact which raises the question whether MVA is the precursor of a more oxidized condensing unit or whether its oxidation state remains unchanged prior to the coupling process. Earlier, we had investigated the utilization of 2-$C^{14}$-5-di-T-MVA for squalene synthesis and observed that the $C_{30}$ hydrocarbon contains the two isotopic markers in the same ratio as MVA.[6] This result eliminated a condensation mechanism of the acyloin type because, on oxidation of $R \cdot CT_2OH$ to $R \cdot COOH$, all the labeled hydrogen would have been removed. Moreover, it was tentatively concluded that MVA could not be utilized by way of aldehydic intermediates because the constant $C^{14}$:T ratio suggested retention of both hydrogen atoms at $C_5$ during the MVA-squalene conversion.[7] Apart from the evidence to be presented below, the following observations are relevant to the question of aldehydic intermediates. Shunk *et al.* have shown that mevaldic acid (3-hydroxy-3-methylglutaraldehydic acid) depresses the incorporation of $C^{14}$-acetate into cholesterol to the same extent as MVA.[8] This finding does not necessarily indicate that mevaldic acid is an obligatory intermediate but can be satisfactorily explained by the assumption that the aldehyde is a precursor of MVA. As reported earlier from this laboratory, dialyzed extracts of autolyzed brewer's yeast catalyze the conversion of MVA to squalene in the presence of ATP, $Mn^{++}$, and pyridine nucleotides.[6] More recently, these extracts have yielded two fractions, A and B, which are inactive separately but will form squalene when combined.[9] Fraction A, in the presence of ATP and $Mn^{++}$, transforms MVA to an intermediate of unknown structure, which in turn is con-

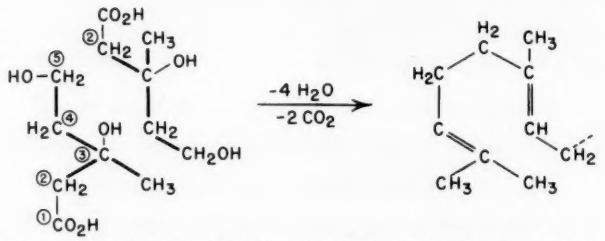



FIG. 1.—Net change in the interaction of two molecules of mevalonic acid

verted to squalene in the presence of pyridine nucleotide and fraction B. The fact that the early transformations of MVA occur in the absence of electron acceptors such as pyridine nucleotides also argues against aldehydes as obligatory intermediates. It is also pertinent that attempts to demonstrate a reduction of DPN by MVA in the same enzyme system have been without success so far.

The structure of the "isoprenoid" condensing units has now been further defined by experiments using a medium of 100 per cent $D_2O$ or using, as the reducing system for the MVA squalene conversion, DPN and deuterioethanol ($CH_3CD_2OH$). In $D_2O$, three to four atoms of D were found to be incorporated per molecule of hydrocarbon, i.e., less than one atom per MVA or isoprenoid condensing unit. Deuterioethanol, in the presence of DPN, furnished less than one atom of D per molecule of squalene (Table 1).

TABLE 1*

INCORPORATION OF D INTO SQUALENE SYNTHESIZED FROM 2-$C^{14}$-MEVALONIC ACID IN $D_2O$, OR IN A MEDIUM CONTAINING 1-$d_2$-ETHANOL

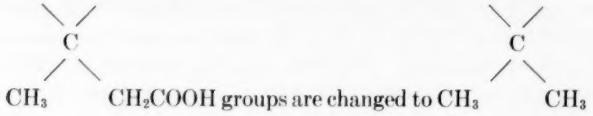
| MEDIUM | MEVALONIC ACID (c.p.m./$\mu$m) | SQUALENE† (c.p.m./$\mu$M) | SQUALENE† (Atom % Excess D) | ATOMS D PER MOLE OF SQUALENE |
|---|---|---|---|---|
| $D_2O$: | | | | |
| 1 | 490 | 43 | 0.11 | 3.6‡ |
| 2 | 490 | 25 | 0.063 | 3.7 |
| 3 | 670 | 42 | 0.073 | 3.5 |
| 4 | 670 | 49 | 0.096 | 3.9 |
| 1-$d_2$-Ethanol | 670 | 58 | 0.007 | 0.3 |

* For these experiments an extract of Baker's yeast[6] was centrifuged at 104,000 × *g*. for 2 hours. The supernatant fluid was dialyzed for 18 hours against three changes of 0.066 *M* $(NH_4)_2HPO_4$ and then lyophilized. For the $D_2O$ experiments this preparation was dissolved in twice its weight of 100 per cent $D_2O$ and lyophilized again. The particles obtained by high-speed centrifugation were washed once with 0.066 *M* $(NH_4)_2HPO_4$, combined with the lyophilized supernatant fraction, and the volume restored to that of the original extract by addition of $D_2O$ or $H_2O$. The water content of the particles is estimated to cause no more than a 2 per cent reduction of the $D_2O$ concentration. Cofactors were added in the following concentrations: ATP and DPN, 1 mg/ml; $Mn^{++}$, 0.001 *M*. The concentration of 2-$C^{14}$-mevalonic acid was $5 \times 10^{-4}$ *M* and of 1-$d_2$-ethanol, when used, 0.1 *M*.

† After dilution of the isolated squalene by non-isotopic carrier.

‡ This sample of squalene was degraded by ozonolysis, according to Cornforth and Popjak (*Biochem. J.*, **58**, 403, 1954). The succinic acid isolated contained 0.5 atoms of D per molecule.

That the over-all transformation of MVA to squalene must be associated with the uptake of hydrogen, regardless of the mechanism involved, is evident from a consideration of the over-all structural changes. First, in those two molecules of MVA which become the terminal isoprene units of squalene the



and hence two atoms of D will be introduced into stable linkages from a $D_2O$ medium. Second, squalene formation from MVA, according to the equation

$$6C_6H_{12}O_4 + 2H \rightarrow C_{30}H_{50} + 6CO_2 + 12H_2O$$

is a reductive process, requiring a net uptake of two atoms of hydrogen. Hence from a $D_2O$ medium either one or two additional atoms of D will be taken up, depending on whether reduction occurs with $H^+$ and $H^-$ or with $2H^+$ and $2e^-$. A minimum uptake of three or four atoms of D is therefore expected from these two processes. The same argument requires that squalene synthesis in the presence of DPN and deuterioethanol (and normal water) take place either without any uptake of D or with the uptake of one atom. From the experimental results obtained with $D_2O$ or deuterioethanol, it is clear that the events just discussed, which are independent of the reaction mechanism, already account fully for the observed hydrogen incorporation. It therefore follows that none of the individual reactions by which MVA is converted to squalene can be associated with any additional uptake of proton or hydride ion from the environment. When applied specifically to the coupling of "isoprenoid" units, this argument leads to the conclusion that both interacting carbon atoms, $C_2$ and $C_5$, retain their hydrogen throughout the condensation process. It is obvious from this argument alone that aldehydic intermediates are disqualified as condensing units.

Another independent line of reasoning leads to the same structural restrictions for the "isoprenoid" intermediate. If the condensing units were carbonyl compounds, the product of the coupling reaction would contain oxygen. For elimination of the oxygen function, dehydration reactions leading to the formation of carbon-carbon double bonds must be invoked. The hydrogen employed for the subsequent reduction of these double bonds could come from only two sources. In processes using flavoprotein as the reductant, hydride and hydrogen ions equilibrate, and hence, if double bonds were being reduced in the present case, the expected uptake of D from $D_2O$ would be two atoms for each condensing unit or a minimum of twelve for every molecule of squalene. In the event that the (hypothetical) double bonds became reduced by hydride transfer from DPND,[10] the number of atoms entering from deuterioethanol should be six. Clearly, the experimental results are not consistent with a condensation mechanism involving such unsaturated intermediates. Therefore, the polyisoprenoid chain must be established without loss and reintroduction of hydrogen at the reacting centers $C_2$ and $C_5$, i.e., by interaction of MVA derivatives which contain $CH_2$ groups at both the $C_2$ and $C_5$ positions. As far as $C_5$ is concerned elimination of water between $C_4$ and $C_5$:$R \cdot CH_2CH_2OH \rightarrow R \cdot CH{=}CH_2$ will yield a methylene group that is reactive. The reactions which lead to a reactive methylene group at the $C_2$ positions must be considered in conjunction with the decarboxylation step. If decarboxylation were to take place prior to condensation and with formation of isopropyl derivatives, one carbon-bound hydrogen would replace each carboxyl group, introducing approximately five atoms of D into squalene from a $D_2O$ environment (Fig. 2, *A*).[11] Since only three to four atoms were incorporated altogether, decarboxylation to isopropyl derivatives is ruled out. Nor is it possible to reconcile the observed uptake of deuterium with a decarboxylation of condensed products (Fig. 2, *B*). However, a reactive methylene group can be established at $C_2$ without concomitant uptake of hydrogen if the removal of the ter-

tiary hydroxyl group takes place simultaneously with the decarboxylation. It will be recognized that the decarboxylation of MVA and the formation of two terminal methylene groups leads to isoprene itself. The conversion of MVA to isoprene without uptake of hydrogen can be formulated as shown in Figure 2, *C*.[12] The essential feature of this mechanism is a decarboxylation in concert with the elimination of the tertiary hydroxyl group which circumvents protonation of the carbon skeleton. Alternative formulations are possible, but these would not alter the principle of the mechanism presented here. For example, it is not critical for the purposes of the present discussion whether elimination of the primary hydroxyl group precedes or follows the concerted decarboxylation.

Squalene, a difarnesyl derivative, is a symmetrical molecule, and hence the reactions which produce the linear polymer from isoprene are likely to be interrupted at the $C_{15}$ stage. To form squalene, two $C_{15}$ units must be aligned in "tail-to-tail" fashion and condensed reductively. On the assumption that it is isoprene itself which condenses, the events leading to a hypothetical sesquiterpenoid intermediate can be formulated as a proton-initiated concerted process (Fig. 3).[13] When proceeding in a medium of $D_2O$, the postulated reactions will introduce a single, stably bound atom of deuterium into each $C_{15}$ unit and therefore account for two of the D atoms found in squalene. The termination of the polymerization process at the sesquiterpenoid stage can be visualized to take one of three courses. The intermediary carbonium ion can be stabilized by (1) proton elimination to form the hydrocarbon farnesene, (2) uptake of $OH^-$ to form the allylic alcohol nerolidol, and (3) isomerization of the carbonium ion by double bond shift and uptake of $OH^-$ to yield farnesol. Farnesene,[14] nerolidol, and farnesol occur in natural sources, and hence the postulated carbonium ion intermediate is eminently suitable as a common precursor.

The coupling of two sesquiterpenoid units to squalene has not been demonstrated in biological systems. Gurin and his collaborators[15] have demonstrated the incorporation of acetate into farnesenic acid in the presence of unlabeled carrier and have thereby shown that liver preparations active in squalene and sterol biogenesis are capable of synthesizing sesquiterpenes. On the other hand, attempts to show a transformation of $C^{14}$-farnesenic acid[16] or of labeled farnesol[17] into squalene or sterols have been unsuccessful.

The earlier reasoning that the formation of saturated carbon chains from carbonyl compounds is necessarily associated with the reduction of double bonds by reaction with either proton or hydride ion is equally relevant to the coupling of farnesyl residues. If, for example, the dihydrotriterpene structure arose by a condensation of two molecules of farnesol, at least three hydrogen atoms would have to enter the center of the squalene chain. While squalene formed in a $D_2O$ medium contains three to four atoms of deuterium, the two carbon atoms in question contain about one-half atom of D, as shown by the analysis of succinic acid after ozonolysis of the hydrocarbon (Table 1). Also, since less than one atom of D is introduced into squalene from the medium containing DPN and deuterioethanol, it is obvious that a condensation of carbonyl compounds followed by reduction cannot be involved. To account for these results, it again becomes necessary to invoke intermediates with terminal methylene groups as reactants in the coupling process. The hydrocarbon farnesene meets these requirements best. A coupling process utilizing

DECARBOXYLATIONS IN $D_2O$

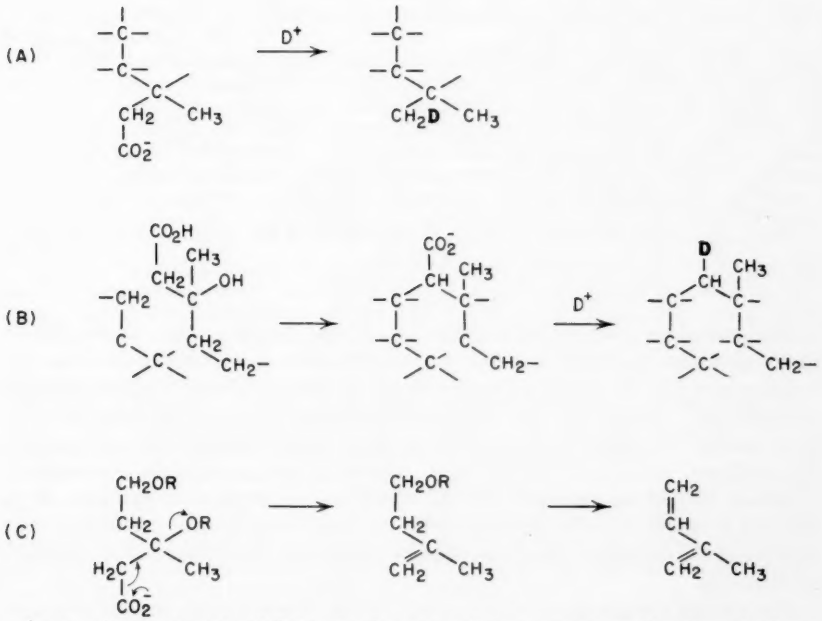



FIG. 2.—Expected uptake of *D* into squalene or squalene precursors resulting from the decarboxylation step. In scheme *C* the hydrogen atoms of the two hydroxyl groups are replaced by *R*, to indicate the possibility of esterification.

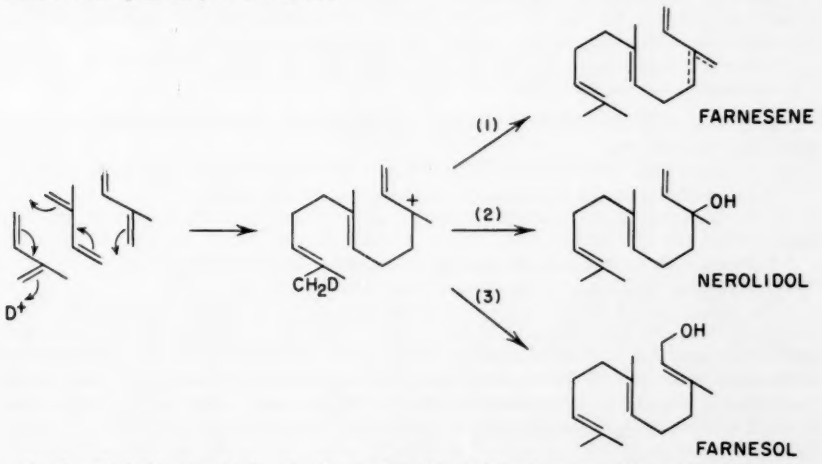



FIG. 3.—Postulated mechanism for the condensation of three isoprene units and transformation of a hypothetical $C_{15}$ carbonium ion to naturally occurring sesquiterpenes.

two molecules of farnesene[18] can be formulated as a concerted process effected by protonation of one $C_{15}$ unit and attack of a hydride ion upon the other (Fig. 4).

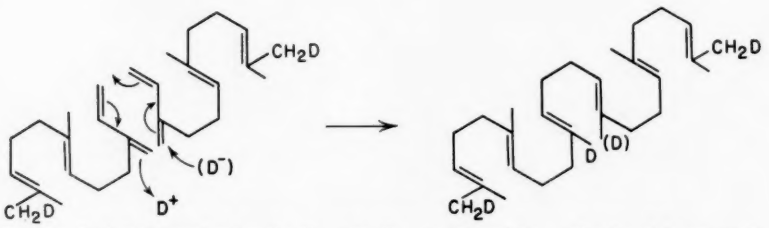



FIG. 4.—Postulated mechanism for the reductive coupling of two molecules of farnesene to squalene.

The postulated mechanisms stipulate an uptake of two protons for the MVA-farnesene transformation and one or two in addition for the farnesene dimerization, in agreement with the observed incorporation of deuterium into squalene from the environment. Finally, it is in favor of our hypothesis that it rationalizes both the total number and the specific location of the double bonds in the end-product. The proposed scheme, which has been developed here specifically for squalene synthesis, should be applicable to the biogenesis of terpenes in general. It is realized, of course, that the evidence so far presented is circumstantial and that the validity of the postulated reactions must be established by further and more direct experiments.

The authors are greatly indebted to the Merck, Sharp, and Dohme Laboratories for generous gifts of labeled mevalonic acid.

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† Holder of a Predoctoral Fellowship of the United States Public Health Services.

‡ American Cancer Society Scholar.

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[7] The tritium-labeled MVA was prepared by reduction of the monomethyl ester of 3$\beta$-acetoxy-3-methylglutaric acid with lithium aluminum tritide. Since the isotopic reagent was not carrier-free, carbon atom 5 of MVA was not totally labeled with T but contained only a small fraction of its hydrogen in the form of the heavier isotope. The expression $R \cdot CT_2OH$ is used, however, to denote that T was randomly distributed between the two hydrogens. Since the chemical rupture of a C-T bond would be slower than that of a C-H bond, it is possible that the relative T concentration increases when MVA is oxidized to mevaldic acid. If, fortuitously, this concentration process balanced exactly the dilution by the hydrogen which is taken up from the medium during the transformation of mevaldic acid to squalene, one would find the same $C^{14}$-T ratio in squalene

as in MVA, even though an aldehyde had been formed as an intermediate. This possibility was considered unlikely and has now been ruled out by the experiments described later in the paper.

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[11] If decarboxylation to isopropyl derivatives took place, the two terminal units would remain as $CH_2D$, but the four internal units would be reconverted into intrachain $—CH_2—$ groups when squalene was formed. Assuming no rate difference between the rupture of C—H and C—D bonds, 4/3 atoms of D would be lost, leaving approximately 5 atoms in squalene.

[12] This discussion has considered transformations involving non-substituted MVA. It has, however, been demonstrated recently that a phosphate ester of MVA is formed from MVA and ATP and that this derivative can be further converted to squalene (T. T. Tchen, *J. Am. Chem. Soc.*, **79**, 6344, 1957). This suggests that one or both hydroxyl groups may be phosphorylated before removal.

[13] It should be pointed out that the formation of the sesquiterpene need not be concerted but could occur in several consecutive steps. For example, the first isoprene molecule, instead of condensing with another isoprene, may interact with an isopentenol phosphate to form geranyl phosphate. The monoterpene derivative could undergo phosphate elimination to form a new carbonium ion for condensation with another $C_5$ unit. This mechanism is mentioned as one of several possible variations of the general scheme shown in Fig. 3.

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[15] F. Dituri, F. A. Cobey, J. V. B. Warms, and S. Gurin, *J. Biol. Chem.*, **221**, 181, 1956.

[16] W. Sandermann and H. Stockmann, *Naturwissenschaften*, **43**, 581, 1956.

[17] H. Rilling and K. Bloch, unpublished work.

[18] The observation that the succinic acid derived from the central carbon atom of squalene contained deuterium, though only a fraction of an atom, is not in accord with the coupling of two farnesene molecules. On the other hand, if one of the two $C_{15}$ units were farnesol, the succinic acid should contain a minimum of 1 atom of D. The presence of the small amount of D at the two central carbon atoms of squalene remains to be explained.

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## *THE INCORPORATION OF AMINO ACIDS INTO RIBONUCLEIC ACID*

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The incorporation of radioactive leucine, valine, and glycine into the ribonucleic acid (RNA) of a soluble enzyme fraction of rat liver has recently been reported by Hoagland *et al.*[1] Ogata and Nohara[2] have studied the incorporation of alanine into RNA. The former authors consider this incorporation to be an intermediate stage in the transfer of labeled amino acids into microsomal protein. This paper describes the fractionation of the crude system for amino acid incorporation into RNA into an activating enzyme fraction and RNA. Both these components are required for incorporation, thus providing a biological assay for functional RNA. Evidence is presented that a specific activating enzyme and a specific RNA are involved in the incorporation of each amino acid.

*Methods and Materials.*—Amino acid-activating enzyme fraction (pH 5 enzyme) was prepared from guinea-pig liver by a modification of the method of Keller and Zamecnik.[3] This was fractionated with aged calcium phosphate gel.[4] The pH 5 enzyme (10 mg/ml) was treated with one-fifth volume of gel (30 mg/ml) and centrifuged, yielding a supernatant fraction ("gel super"). The precipitated gel was eluted with 2.0 *M* phosphate buffer, pH 8.1, centrifuged, and the eluate dialyzed ("gel eluate"). $AS_{30-60}$ fraction is that fraction of the "gel super" which precipitates between 30 and 60 per cent saturation with ammonium sulfate at pH 7.5. Treatment with ribonuclease (Table 2) involved preincubation with 0.1 μg. of crystalline ribonuclease for 10 minutes at 37°, then dilution with the assay constituents and the usual assay. The low level of ribonuclease used and the dilution minimize ribonuclease action during the assay.

The reaction mixture for incorporation contained: 8 mg. of the pH 5 fraction or the equivalent aliquot; 10 μmoles of potassium adenosine triphosphate (ATP), pH 7.5; 10 μmoles of magnesium chloride; 100 μmoles of tris buffer, pH 7.5; either 0.05 μmoles of uniformly labeled $C^{14}$-L-leucine (1,100 counts/min/mμmole), 0.025 μmoles of uniformly labeled $C^{14}$-L-tyrosine (3,000 counts/min/mμmole), or 0.025 μmoles of uniformly labeled $C^{14}$-L-threonine (2,500 counts/min/mμmole); and water to a final volume of 1.4 ml. The mixture was incubated for 10 minutes at 37°, precipitated with 8 volumes of 3.5 per cent trichloracetic acid, and washed as described by Hoagland *et al.*[1] Inert protein was added before precipitation (when needed) to make the final weight of protein plus RNA counted approximately 10 mg. The pyrophosphate exchange assay for activating enzyme has been described.[5] All samples were counted with a Nuclear "Micromil" window gas-flow counter.

*Experiments and Results.*—Table 1 shows incorporation of $C^{14}$-labeled tyrosine and leucine into the pH 5 enzyme fraction of guinea-pig liver. Incorporation at zero time or in the absence of ATP is less than 5 per cent of that of the complete system. Enzyme preincubated for 10 minutes at 25° with 0.2 μg. of ribonuclease/ml is also inactive. Incorporated amino acid is released by ribonuclease and by dilute alkali, indicating binding to RNA.

TABLE 1

FRACTIONATION OF INCORPORATING AND ACTIVATING SYSTEMS

| FRACTION | AMINO ACID INCORPORATION Total Activity in mμmoles* Tyrosine | Leucine | PYROPHOSPHATE EXCHANGE Total Activity in mμmoles* Tyrosine | Leucine |
|---|---|---|---|---|
| pH 5 enzyme | 0.21 | 0.89 | 700.0 | 1,200.0 |
| "Gel super" | 0.01 | 0.05 | 200.0 | 420.0 |
| "Gel eluate" | 0.16 | 0 | 350.0 | 0 |
| "Gel super" plus "gel eluate" | 0.14 | 0.62 | — | — |
| Heated "eluate"† | 0.01 | 0 | 0 | 0 |
| Heated "eluate" plus "gel super" | 0.15 | 0.68 | — | — |
| Heated "gel super" plus "eluate" | — | 0.07 | — | — |
| $AS_{30-60}$ plus heated "eluate" | 0.18 | 0 | 65.0 | 0 |

* The results are given for 1 ml. of pH 5 enzyme or its equivalent, so that the recovery of enzymatic activity in the various fractions can be calculated from the data. The spaces with dashes indicate "not measured."
† Heated at 80° for 10 minutes.

Fractionation of the crude pH 5 enzyme with calcium phosphate gel gives two fractions—the "gel eluate" and the "gel super" (Table 1). Neither fraction alone is active for leucine incorporation, but almost full activity is restored when the fractions are recombined. The values for pyrophosphate exchange (Table 1)

indicate that activating enzyme for leucine is present only in the "gel super" and not in the "gel eluate." The heat-stable component of the "gel eluate" is therefore not required for activation but is required in addition to activating enzyme for incorporation activity.

The "eluate fraction" alone incorporates tyrosine in good yield (Table 1). Tyrosine-activating enzyme is present in this fraction, however. The activating enzyme in the "eluate" is destroyed by heating, and, since tyrosine-activating enzyme is also present in the "gel super," "heated eluate" plus "gel super" restores incorporating activity for tyrosine (Table 1). Thus the tyrosine-incorporating system also consists of a heat-stable component and an activating enzyme fraction but differs from the leucine system in the adsorption of most of the tyrosine-activating enzyme by the gel. Tyrosine-activating enzyme of hog pancreas is also separated from other activating enzymes by this procedure.[6] The tyrosine-incorporating system of the "gel eluate" is purified twentyfold over the original pH 5 enzyme because of the separation of the activating enzyme by this step. Fractionation of the "gel super" with ammonium sulfate yields a tyrosine-activating enzyme free of leucine-activating enzyme, and this fraction will restore only tyrosine incorporation to the heated "eluate" (Table 1). Similarly, a leucine-activating fraction, free of tyrosine-activating enzyme, will restore only leucine incorporation (data not shown). These results provide, for the first time, direct evidence that specific amino acid-activating enzymes are required for the incorporation of specific amino acids but do not exclude the participation of other factors in the activating enzyme fractions. The occurrence of activating enzymes in the pH 5 fraction[7] and the correlation between activation and incorporation of tryptophan analogues[8] have previously been cited as evidence for the role of activating enzymes in amino acid incorporation.

Several considerations suggest that the "eluate" fraction (Table 1) contains RNA and that this is the "acceptor" for the activated amino acid. Thus the "eluate" contains 400–500 $\mu$g. of RNA/ml[9] and 500–600 $\mu$g. of protein.[10] This fraction is inactivated by ribonuclease, in contrast to the activating enzyme fraction (Table 2),

TABLE 2

RECOMBINATION OF ACTIVATING ENZYME WITH RNA

| FRACTION | AMINO ACID INCORPORATED* Total Activity in mμmoles Tyrosine | Leucine |
|---|---|---|
| Ribonuclease-treated "gel super" plus "eluate" | — | 0.55 |
| Ribonuclease-treated "gel eluate" plus "super" | — | 0.06 |
| RNA from "gel eluate" plus "super" | 0.12 | 0.62 |
| RNA from microsomes plus "gel super" | 0.01 | 0.03 |

* These results are comparable with those given in Table 1, although different enzyme preparations were used. Neither the "gel super" used for these experiments nor the RNA showed incorporation separately.

which is not. Finally, treatment of the "gel eluate" with phenol by a procedure patterned after the method of Kirby[11] yields RNA, which can substitute completely for the "eluate" (Table 2). RNA prepared by phenol treatment from the "eluate fraction" is, in fact, as active for incorporation as the original material. The activity shown in Table 2 represents the yield of RNA obtained by the extraction procedure rather than any inactivation. This yield ranges from 60 to 90 per cent

of the RNA present in the particular fraction. RNA prepared from guinea-pig liver microsomes by the same procedure is inactive (Table 2). Various other RNA preparations, such as those from virus and yeast, are also inactive. However, RNA from the pH 5 enzyme fraction of rat liver is fully active with activating enzymes from guinea-pig liver. RNA prepared by extraction with hot 2.0 *M* sodium chloride[12] also possesses high incorporation activity in this system.

Finally, it appears that specific RNA's are needed for the incorporation of individual amino acids. Under the usual conditions, there is a definite upper limit of incorporation for each amino acid. For leucine this is regularly four- to fivefold greater than for tyrosine (Table 1). For threonine, it is threefold greater than for tyrosine. Table 3 shows that incorporation of two amino acids—tyrosine and threonine—is additive. Furthermore, addition of non-radioactive threonine does not inhibit incorporation of radioactive tyrosine and vice versa. These experiments were done under conditions of maximal incorporation, so that available RNA is the limiting factor. Since each amino acid is incorporated independently, each must, apparently, be incorporated into a specific RNA. Preliminary fractionation studies appear to support this conclusion, since a "gel super" containing leucine-acceptor RNA, but not tyrosine-acceptor RNA, has been prepared. These experiments suggest that the isolation of specific RNA and the correlation of RNA structure with a biological activity are now possible.

TABLE 3

TYROSINE AND THREONINE COMPETITION

| Additions | Amino Acid Incorporated* Total Activity in mμmoles |
|---|---|
| $C^{14}$-tyrosine | 0.15 |
| $C^{14}$-threonine | 0.44 |
| $C^{14}$-tyrosine plus $C^{14}$-threonine | 0.56 |
| $C^{14}$-tyrosine plus cold threonine | 0.15 |
| $C^{14}$-threonine plus cold tyrosine | 0.46 |

* The enzyme fraction used was the "gel eluate" equivalent to 1 ml. of original pH 5 enzyme. The standard assay conditions were used. The non-radioactive (cold) amino acids were added at the level usually used in the assay.

*Summary.*—A soluble enzyme system which incorporates labeled amino acid into RNA, has been separated into two components. These are activating enzyme fraction and RNA acceptor. Both are needed for incorporation of amino acids. Activating enzyme fractions specific for tyrosine and leucine are required for the incorporation of the respective amino acid. The RNA acceptors appear also to be specific for each amino acid.

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† National Science Foundation Postdoctoral Fellow, summer, 1957.

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## *FLUORESCENCE STUDIES OF COENZYME BINDING TO DEHYDROGENASES**

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The mechanism of binding of pyridine coenzymes to dehydrogenases has been studied principally by two techniques. One of these is the ultracentrifugal method,[1] and the other, which has been used more extensively, is the spectrophotometric measurement of characteristic bands of the enzyme-coenzyme complex. Theorell and Bonnichsen[2] reported a shift in the spectrum of reduced diphosphopyridine nucleotide (DPNH) in the presence of horse liver alcohol dehydrogenase, and Boyer and Theorell[3] have also reported a shift in the fluorescence spectrum of DPNH upon the addition of liver alcohol dehydrogenase.

Recently, studies in coenzyme binding to dehydrogenases have been facilitated by the preparation of analogues of DPN in which the nicotinamide moiety is replaced by other pyridine derivatives.[4] While Chance and Nielands[5] could demonstrate a spectral shift of DPNH with beef heart lactic dehydrogenase (LDH) only at low temperatures and with a "sensitive" spectrophotometer, Kaplan, Ciotti, and Stolzenbach[6] have demonstrated a shift of the reduced acetyl pyridine analogue of DPN (APDPNH) at room temperature. This report deals with the fluorescence changes associated with the addition of APDPNH to crystalline beef heart LDH.

*Methods and Materials.*—The beef heart and rabbit muscle lactic dehydrogenase were obtained by the respective methods of Straub[7] and Kornberg.[8] Horse liver ADH was a crystalline product prepared by the procedure of Bonnichsen and Brink.[9] The coenzyme analogue was prepared as described by Kaplan and Ciotti.[10]

Sodium *p*-chloromercuribenzoate (PCMB) was obtained from the Sigma Chemical Company and purified as described by Boyer.[11]

The fluorescence spectra were studied with the Aminco-Bowman spectrophotofluorometer manufactured by the American Instrument Company. This fluorometer has been described by Bowman *et al.*[12]

*Results.*—When a solution of APDPNH is excited by ultraviolet light having a wave length of 365 m$\mu$, a fluorescence with its maximum at 480 m$\mu$ is emitted. Upon the addition of small quantities of crystalline beef heart LDH, the maximum

is shifted to 460 mμ, accompanied by a very pronounced increase in intensity of fluorescence (Fig. 1). A similar shift was not observed when DPNH was used, which is in accordance with the spectrophotometric results of Kaplan *et al.*[6]

Crystalline beef heart LDH itself emits an intense fluorescence at 350 mμ when excited by 288-mμ light. This does not appear to result from any contaminant of the sample. The fluorescence pattern cannot be reproduced by a mixture of aromatic amino acids which were studied. When the enzyme is treated under denaturation conditions, the intensity of the 350-mμ fluorescence is considerably reduced. Boiling of the enzyme for 1 minute in buffer or dissolving it in 0.1 *N* HCl resulted in loss of fluorescence. The denaturing action of amides was studied by adding the enzyme to various concentrations of urea. In concentrations of 1–4 *M*, urea is without effect on the intensity, whereas in 5 *M* urea the fluorescence of the enzyme is lost. This suggests that the 350-mμ fluorescence is a property related to the conformation of the native protein.

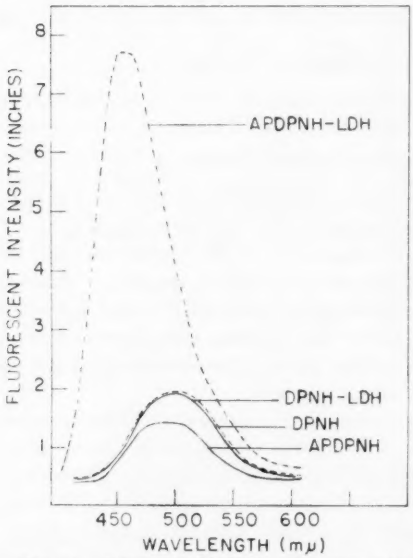


FIG. 1.—Shift of APDPNH spectrum with beef heart LDH. Excitation wave length is 365 mμ. The reaction mixture contained 1.0 μg. APDPNH, 6.4 μg. beef heart LDH and brought to a final volume of 1.0 ml. with 0.1 *M* phosphate buffer, pH 7.0. The same quantities were used with DPNH.

The addition of small quantities of APDPNH to the beef heart lactic dehydrogenase results in a gradual decrease in the 350-mμ intensity, with the simultaneous appearance of the fluorescence peak at 450 mμ. The intensities of both peaks remain unchanged beyond the addition of 2 micromoles of APDPNH per micromole of beef heart lactic dehydrogenase (see Fig. 2). Since the fluorescence of APDPNH is extremely weak in the absence of the enzyme when activated at 288 mμ, the 450-mμ fluorescence indicates the formation of a LDH-APDPNH complex.

The absence of any observable shift in DPNH absorption in the presence of LDH was mentioned above. The addition of DPNH to lactic dehydrogenase is not accompanied by the appearance of a fluorescence peak at 450 mμ when activated at 288 mμ, even though DPNH per se is strongly fluorescent.

The oxidized form of the coenzyme analogue, APDPN, will not bind to the beef heart enzyme, and there is no apparent fluorescence at 450 mμ when the oxidized analogue and the lactic dehydrogenase are mixed. When excess pyruvate is added to the LDH-APDPNH complex having the fluorescence pattern indicated by the dotted line in Figure 2, the 450-mμ peak disappears, and the intensity of the 350-mμ peak increases. The reverse reaction—addition of lactate to a mixture of LDH and APDPN—gives the same fluorescence pattern as that obtained by direct addition of APDPNH to the beef heart enzyme. When lactate or pyruvate is added to the

enzyme in the absence of coenzyme, the 350-mμ fluorescence remains unchanged. This is in agreement with the suggestion by van Eys *et al.*[13] that a ternary complex is formed during enzymic activity between the enzyme, coenzyme, and a nucleophilic compound.

The enzymatic activity of beef heart LDH has been inhibited by incubation with with $10^{-3}$ *M* *p*-chloromercuribenzoate (PCMB).[14] Such treatment has little effect on the fluorescence intensity of the enzyme. When APDPNH is added in small increments to the PCMB-treated enzyme, the intensity of the 350-mμ fluorescence decreases as it did with the uninhibited LDH, but the 450-mμ peak does not appear. The inhibiting action of PCMB may be reversed by the addition of excess cysteine to the solution of altered enzyme. Under these conditions the 450-mμ fluorescence appears, as the sulfhydryl groups of the protein are free to bind the APDPNH.

Another group which has been implicated in the enzymatic activity of LDH has been the amino group. Nygaard[15] demonstrated that the enzymatic activity of lactic dehydrogenase is inhibited after treatment with acetic anhydride. The fluorescent behavior of the crystalline enzyme after acetic anhydride treatment is similar to that which is obtained with the denatured enzyme. It thus appears that loss of enzymatic activity induced by acetic anhydride is associated with the loss of the fluorescence at 350 mμ.

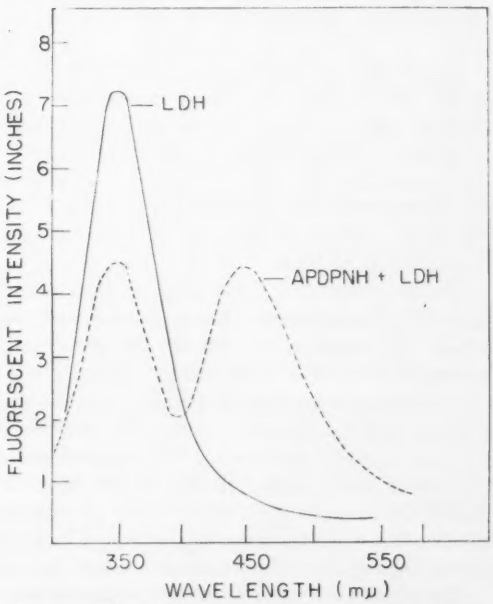


FIG. 2.—Fluorescence spectrum of LDH and LDH-APDPNH complex activated at 288 mμ. Solid line represents 6.4 μg. LDH in 1.0 ml. of 0.1 *M* phosphate buffer. pH 7.0. Dotted line shows the spectrum of the same amount of LDH in combination with 0.5 μg. APDPNH.

The fluorescent behavior of other dehydrogenases has been studied. The shift in the fluorescence peak of DPNH upon addition of horse liver alcohol dehydrogenase was reported by Boyer and Theorell.[3] Using DPNH, relatively large quantities of the liver enzyme were required to produce an observable shift of the fluorescence maximum with a slight intensification; however, only a few gamma of the enzyme are needed to produce a similar shift, accompanied by pronounced intensification with the reduced acetyl pyridine analogue of DPN.

Rabbit skeletal muscle lactic dehydrogenase has no effect on the fluorescence maximum of either APDPNH or DPNH, which is consistent with the spectrophotometric results of Kaplan *et al.*[6]

*Discussion.*—The shift in the fluorescence maximum of APDPNH and the increased intensity upon combination with the beef heart enzyme and horse liver ADH suggest that this method is most suitable for the study of pyridine nucleotide binding to dehydrogenases, since changes can be detected with only a few gamma of protein.

The strongly fluorescent 350-m$\mu$ peak of the enzyme appears to depend upon the conformation of the protein in its native form. The reversible and irreversible denaturation of the enzyme results in the disappearance of the 350-m$\mu$ fluorescence. The appearance of the 450-m$\mu$ peak accompanying the addition of APDPNH to the beef heart enzyme seems to depend upon formation of the LDH-APDPNH complex. Alteration in the structure of either the coenzyme or the enzyme which interferes with formation of the complex also appears to inhibit the 450-m$\mu$ fluorescence.

The simultaneous decrease of the 350-m$\mu$ fluorescence intensity with the increase in the 450-m$\mu$ peak during coenzyme binding leads one to speculate as to the changes which occur to the protein during enzymatic activity. From some interesting fluorescence experiments in ordinary ice, Szent-Györgyi[16] has suggested that some biological phenomena may be a response to a rearrangement in the structure of the water envelope of proteins. The presence of structured water of "icebergs" around the protein molecule has been emphasized by Jacobsen[17] and more recently by Klotz.[18, 19]

The fluorescence of the crystalline beef heart lactic dehydrogenase may be enhanced by the presence of the structured water, and conditions such as heat or acid which may transform the ordered water into a more random distribution of water molecules may cause a decrease in intensity of fluorescence. Urea, which has excellent hydrogen-bonding properties, would randomize the "frozen" water. Similar changes may be assumed to occur after addition of APDPNH, since here again the 350-m$\mu$ intensity decreases. The reduced coenzyme appears to randomize the structured water during binding to the protein. The formation of the enzyme-APDPNH complex is accompanied by the fluorescence emission at 450 m$\mu$ and indicates that when the reduced analogue is bound to the enzyme, the reduced acetyl pyridine grouping is much more fluorescent than when it is unbound.

The structured water lattice may also be responsible for the relative inactivity of sulfhydryl groups toward reagents which require an incubation period for reaction. The reagent must first dissolve in the structured water, which does not necessarily randomize the rigid structure. Thus PCMB-treated lactic dehydrogenase emits the fluorescence at 350 m$\mu$ with the same intensity as the untreated enzyme. The reduced coenzyme analogue, APDPNH, still has the capacity to randomize the frozen water, resulting in the decrease in 350-m$\mu$ intensity, but its binding to the protein is inhibited, as indicated by the absence of 450-m$\mu$ fluorescence. Removal of the inhibitor by addition of excess cysteine allows APDPNH to bind and hence the appearance of the 450-m$\mu$ fluorescence.

The data suggest that the alteration in protein structure which accompanies the formation of an enzyme-coenzyme complex may be similar to the structural changes which occur during denaturation. The decreased rate of denaturation of dehydrogenases in the presence of reduced coenzymes supports this view.

*Summary.*—The fluorescence spectrum of reduced DPN is not altered when

added to crystalline beef heart lactic dehydrogenase. However, when the reduced acetyl pyridine analogue of DPN is added to this crystalline enzyme, a very marked increase in the intensity of the fluorescence of the reduced analogue is observed. This increase is associated with a shift in the fluorescence maximum of the analogue and is dependent on the amount of lactic dehydrogenase added. The differences observed between reduced DPN and the reduced acetyl pyridine analogue are in accordance with the spectrophotometric observations. Using the fluorescence procedure, this binding of lactic dehydrogenase with the reduced coenzyme analogue can be determined with only a few gamma of protein. Crystalline beef heart lactic dehydrogenase gives a fluorescence maximum at 350 m$\mu$ when activated at 288 m$\mu$. This fluorescence pattern cannot be reproduced by a mixture of aromatic amino acids. Treatment by agents which produce reversible and irreversible denaturation of the enzyme causes a loss of the fluorescent characteristics of the enzyme. The 350-m$\mu$ maximum of the lactic dehydrogenase is also decreased by the addition of the reduced acetyl pyridine analogue of DPN.

The significance of the fluorescence changes associated with coenzyme binding is briefly discussed.

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# *INACTIVATION OF HUMAN ERYTHROCYTE AGGLUTINOGENS M AND N BY INFLUENZA VIRUSES AND RECEPTOR-DESTROYING ENZYME*

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The ability of influenza viruses and receptor-destroying enzyme from *Vibrio cholerae* (R.D.E.) to remove the serologically specific heterogenetic mononucleosis receptor from sheep erythrocytes has recently been reported.[1] It was of interest, therefore, to investigate the effect of influenza viruses and R.D.E. upon serologically recognized receptors of human erythrocytes.

The present paper reports on the selective inactivation of blood group agglutinogens M and N from human erythrocytes by two strains of influenza virus type A —swine influenza virus strain $S_{15}$ and the Melbourne strain—by the Lee strain of influenza virus type B, and also by R.D.E.

## MATERIALS AND METHODS

*Enzymatically Active Agents.*—Influenza viruses were propagated by inoculation into the allantoic cavities of 10-day-old embryonated hen eggs and incubation for 48 hours at 37° C. The infected allantoic fluid was harvested after chilling the eggs. The Melbourne strain of type A, the $S_{15}$ strain of swine influenza virus, and the Lee strain of type B virus, respectively ($1 \times 10^8$ to $1 \times 10^9$ $I.D._{50}$ of virus per ml. of allantoic fluid) were used after purification and four- to eightfold concentration by absorption on and elution from chick erythrocytes. To 50–100 ml. of freshly harvested infected allantoic fluid chilled to 4° C. was added one-tenth volume of washed and packed precooled chick erythrocytes. The mixture was kept 1 hour at 4° C. and repeatedly shaken. The suspension was then centrifuged in the cold, the supernatant fluid discarded, and the cells washed once with cold saline. The packed erythrocytes were resuspended in 15–25 ml. saline, and the virus was eluted at 37° C. during a 10–12-hour period. Subsequently, the chicken erythrocytes were removed by centrifugation, and the virus suspension was used for the enzyme experiments. Occasionally, the virus suspension was reused following additional absorption on and elution from chick erythrocytes after it had acted upon human erythrocytes.

R.D.E. from *Vibrio cholerae* (lot No. 18 Behringwerke, Germany) was employed in concentration of about 200 units/ml. A trace of $CaCl_2$ was added.

*Erythrocytes.*—Erythrocytes from thirteen different donors possessing the following antigens on their surface were used: (1) O, CDE/e, NN, P; (2) O,cDE/ce, MM, P; (3) $A_1$, CDe/e, NN, P; (4) B,CDE/Ce, MM, S; (5) O,CDe/e, MN; (6) O,CDE/ce, MM, P, S; (7) O,CDe/ce, NN, P; (8) $A_1$cde/cde, MM, P, S; (9) $A_1$B,CDe/e, MM; (10) O,CDe/Ce, MN; (11) $A_1$B,cDE/c, NN; (12) O,cDE/cE and (13) O,cde/cde, NN, Kk. Wherever possible, erythrocytes homozygous for

the antigen under study were employed. Erythrocytes from citrated human blood of the desired group, stored for less than 1 week, were washed three times with saline and were used as a 2 per cent suspension in all serological experiments. Heparinized chicken erythrocytes, 20–24 hours old, were employed after two washings with saline for the concentration and purification of influenza viruses.

*Antisera.*—The following sera were procured in this laboratory: human "natural" anti-A and B from blood group O person; human "immune" anti-A from B person; human "immune" anti-B from A person; eel anti-H(O) absorbed prior to use with $A_1B$ cells; human "natural" anti-M; human immune anti-P; and human immune anti-K. The following sera were obtained from Ortho Research Foundation, Raritan, N.J.: one human "immune" anti-B; three human anti-M; one human anti-P; one human anti-S; three rabbit anti-M; three rabbit anti-N; two human anti-D; two human anti-C; two human anti-E; two human anti-c; and one human anti-e. A saline agglutinating anti-S serum was generously donated by Dr. R. E. Rosenfield, Department of Health, New York City.

All agglutinins except anti-c, anti-K, and one anti-S were fully active when tested on red cells in saline suspension. The action of these antibodies was investigated by the indirect Coombs test. The Coombs serum was an antihuman globulin serum (Ortho Lot R7093-L). This diluted serum did not contain demonstrable "T" agglutinins and was used unabsorbed.

All sera containing anti-A or anti-B agglutinins or both were tested against O cells only, unless agglutinogen A or B was being investigated. Except for sera containing anti-A and anti-B agglutinins, no serum contained demonstrable agglutinins against more than one agglutinogen of the intact erythrocyte.

*Solutions.*—Buffered saline solution (0.85 per cent NaCl, 0.025 molar phosphate pH 7.2) was used throughout. In all enzymatic experiments, both penicillin and dihydrostreptomycin were added in final concentration of 200 units/ml.

*Treatment of Human Erythrocytes with Influenza Viruses and R.D.E.*—Washed and packed human erythrocytes were added to a fivefold volume of virus suspension and incubated with repeated shaking for 8–20 hours at 37° C. Subsequently, the red cells were separated and washed two to three times with saline at room temperature (22°–25° C.). When R.D.E. was used instead of influenza viruses, similar conditions were maintained, except that the incubation period varied from 2 to 7 hours. In order to observe the influence of prolonged incubation on erythrocyte receptors, tubes containing uninoculated allantoic fluid and erythrocytes to which only buffered saline with antibiotics had been added were included in the studies. Influenza virus preparations and R.D.E. which had been boiled for 30 minutes to inactivate them were also tested.

*Absorption of Serum.*—An equal amount of serum and packed human erythrocytes treated with virus or R.D.E. as described above was mixed well and kept at room temperature (22°–25° C.) for 2 hours, with frequent shaking during the first 90 minutes. After centrifugation the erythrocytes were discarded, and the serum was used for titration. Eel anti-H(O) serum was absorbed twice. Within each experimental series aliquots of erythrocytes from the same donor treated with the same enzymatically active agent were used for both absorption of sera and titration of agglutinins. Absorption of anti-M and anti-N sera with native MM and NN

erythrocytes was also carried out in order to assess the degree of removal of the corresponding antibodies.

*Indirect Coombs Test.*—This test was performed according to the procedure given by the Ortho Research Foundation. After incubation of a 2 per cent erythrocyte suspension with incomplete antibody for 1 hour, the red cells were washed three times. The supernatant fluid was removed, 0.1 ml. antihuman globulin serum was added, and the mixture was centrifuged at 1,000 r.p.m. for 1 minute. Readings of agglutination were made with the naked eye. In each Coombs test a positive and a negative control were included.

*Titration of Agglutinins.*—The method of titration and interpretation of agglutination reactions have been described previously.[1, 2] In the present experiments the volume of each reagent in the test was 0.1 ml. A different pipette was used for each tube in a titration series. Incubation was at about 22° C. except for tests with anti-P, which were incubated at 4° C., and tests with anti-S, which were incubated at 37° C. Agglutination reactions were read with the naked eye at 2 hours and again at 10 hours of incubation after the addition of erythrocytes, except for tests with animal anti-M and anti-N sera, which were read after 30–40 minutes and again after 2 hours of incubation. Each titration included a control of a suspension of intact erythrocytes and also a saline suspension of red cells treated with the enzymatically active agent.

*Hemagglutination Inhibition.*—The method for studying inhibition of hemagglutination has been described previously.[1, 2] Twofold geometrical dilutions of inhibitor were incubated with four minimum hemagglutinating doses of serum for about 10 hours at 4° C. The appropriate erythrocytes were then added. The results were read after an additional incubation of 2 hours at room temperature (22°–25° C.), except in those instances where animal anti-M and anti-N sera were employed, when the test was read 30–40 minutes after the addition of erythrocytes.

*Preparation of Enzymatic Digests from Erythrocytes.*—The supernate of enzyme-treated erythrocytes was used either unboiled or after exposure in a boiling water bath for 20 minutes, because M and N agglutinogens are considered to be heat-stable.[3] Insoluble material was separated by centrifugation and tested as a suspension. The suitably concentrated water-soluble material was tested as such and also after dialysis in cellophane casing (Visking, average pore size 48 Å) for 72 hours at 4° C. against 60 volumes of distilled water, which was changed four times.

The color reactions employed in this investigation were the same as those employed previously[1] for the demonstration of the presence of carbohydrate (Molisch and Anthrone reactions) and for neuraminic acid and its derivatives (Morgan-Elson reaction without alkali, and Bial's test, neuraminic acid modification).

## RESULTS

Tables 1 and 2 summarize the serological observations. Table 1 shows that treatment of human erythrocytes with swine influenza virus inactivates the antigens M and N, while none of the other blood group agglutinogens tested was demonstrably affected. The Melbourne strain of type A influenza virus and the Lee strain of type B influenza virus affected the erythrocytes in the same way. The preparations of the Lee strain appeared to be weaker in activity especially against N than the strains of type A virus.

TABLE 1

EFFECT OF $S_{15}$ STRAIN OF SWINE INFLUENZA VIRUS UPON BLOOD GROUP AGGLUTINOGENS OF HUMAN ERYTHROCYTES

| SERUM | AGGLUTINOGEN TESTED FOR | ERYTHROCYTE LOT NO. | SERUM UNABSORBED | | SERUM ABSORBED WITH VIRUS-TREATED ERYTHROCYTES | |
|---|---|---|---|---|---|---|
| | | | Agglutination of Human Erythrocytes* | | | |
| | | | Native | Virus-treated | Native | Virus-treated |
| Anti-A Human "immune" (5/57) | $A_1$ | III | 256 | 256 | 2 | 4 |
| Anti-A Human "natural" O person (6780/56) | $A_1$ | III | 8 | 8 | <1 | <1 |
| Anti-A Human "natural" O person Spr. | $A_1$ | III | 32 | 32 | <1 | <1 |
| Anti-B Human "immune" (12/55) | B | IV | 64 | 64 | <1 | <1 |
| Anti-B Human "immune" Ortho 9059-2 | B | IV | 128 | 128 | <1 | <1 |
| Anti-H(O) Eel (1956) | O(H) | V | 64 | 256 | 2 | 2 |
| Anti-A & B Human V "natural" O person (6780/56) | $A_1B$ | XI | 8 | 8 | <1 | <1 |
| Anti-M Human Ortho R8064 | M | VI | 512 | 8 | 64 | <1 |
| Anti-M Human Ortho T2015 | M | VI | 32 | 4 | 32 | <1 |
| Anti-M Human Cald. | M | IV | 32 | 8 | 32–64 | <1 |
| Anti-M Animal Ortho 16 | M | II | 16 | 4–8 | 16 | <1 |
| Anti-M Animal Ortho 11, 13 | M | IV | 8 | 4 | 4 | <1 |
| Anti-N** Animal Ortho 6, 9 | N | III | 16 | 8 | 8 | <1 |
| Anti-N** Animal Ortho 12 | N | III | 16 | 4 | 8 | 1 |
| Anti-M Human Cald. | MN | V | 16 | 16 | 16 | <1 |
| Anti-N Animal Ortho 12 | MN | V | 8 | 2 | 8 | <1 |
| Anti-S Human saline Fa 2-214 | S | VI | 4 | 4 | <1 | <1 |
| Anti-S Human incomplete Ortho | S | IV | 8 | 16 | <1 | <1 |
| Anti-P Human Glai. | P | I | 4 | 8 | <1 | <1 |
| Anti-P Human Ortho | P | VI | 16 | 16–32 | <1 | <1 |
| Anti-K Human incomplete Mor. | K | XIII | 8 | 8–16 | <1 | 1 |
| Anti-C Human Ortho 3080 | C | III | 8 | 32–64 | <1 | 1–2 |
| Anti-C Human Ortho 3080 | C | IV | 8 | 16 | <1 | 1 |
| Anti-D Human Ortho R1208 | D | III | 4 | 8 | <1 | <1 |
| Anti-D Human Ortho R1207 | D | I | 8 | 16 | <1 | 2 |
| Anti-E Human Ortho R4056 | E | IV | 8 | 8 | <1 | 1 |
| Anti-E Human Ortho R4055 | E | II | 8 | 16 | <1 | 2 |
| Anti-c Human incomplete Ortho R 0024, 0027 | c | VIII | 16 | 32 | <1 | <1 |
| Anti-e Human Ortho | e | VIII | 16 | 16 | <1 | <1 |

* Expressed as reciprocal of serum dilution.

** "Essentially the same results were obtained with plant anti-N (titer 1:32 at 37° C.) from *Vicia graminea* seeds."

A comparison of the data in the fourth and sixth columns of Table 1 shows that absorption of human sera with virus-treated red cells removes all agglutinins against normal erythrocytes, except those against the antigens M and N, regardless of whether the erythrocytes are homozygous or heterozygous with respect to blood group antigens M and N. The anti-M and anti-N titers against intact erythrocytes are unchanged by absorption with virus-treated red cells (fourth and sixth columns, Table 1). This indicates inactivation of M and N antigens of the virus-treated red cells used for absorption. Untreated MM and NN erythrocytes, on the other hand, removed the homologous antibodies in one absorption. Inactivation of M and N agglutinogens by influenza virus is also demonstrated in the last column of

TABLE 2

EFFECT OF "RECEPTOR-DESTROYING ENZYME" UPON BLOOD GROUP AGGLUTINOGENS OF HUMAN ERYTHROCYTES

| SERUM | | AGGLUTINOGEN TESTED FOR | ERYTHROCYTE LOT NO. | SERUM UNABSORBED | | SERUM ABSORBED WITH R.D.E.-TREATED ERYTHROCYTES | |
|---|---|---|---|---|---|---|---|
| | | | | Agglutination of Human Erythrocytes* | | | |
| | | | | Native | R.D.E.-treated | Native | R.D.E.-treated |
| Anti-A | Human "immune" (5/57) | $A_1$ | III | 512 | 1,024 | 4 | 4–8 |
| | Human "natural" (6780/56) | | III | 8–16 | 16 | <1 | <1 |
| Anti-B | Human "immune" (12/55) | B | IV | 128 | 256 | <1 | <1 |
| | Human "immune" Ortho 9059-2 | | IV | 128 | 256 | <1 | 1 |
| Anti-H(O) | Eel (1956) | O(H) | VI | 64 | 512 | <1 | 2 |
| Anti-A | Human "immune" (5/57) | $A_1B$ | IX | 256 | 256 | 1 | 4 |
| Anti-B | Human "immune" (12/55) | | IX | 64 | 64 | <1 | <1 |
| Anti-M | Human Ortho R8064 | M | VI | 512 | 8 | 512 | <2 |
| | Human Ortho T2015 | | VI | 64 | 8 | 64 | <1 |
| | Human Ortho 16 | | IV | 32 | 16 | 32 | <1 |
| | Animal Ortho 11 | | IV | 32 | 8 | 16 | <1 |
| | Human Cald. | | II | 32 | 32 | 32 | <1 |
| Anti-N | Animal Ortho 6, 9 | N | III | 8 | 4 | 2–4 | <1 |
| | Animal Ortho 6, 9 | | VII | 16 | 4–8 | 8 | <1 |
| | Animal Ortho 12 | | VII | 8 | 8 | 8 | <1 |
| Anti-M | Human Cald. | MN | X | 16–32 | 32 | 32 | <1 |
| Anti-N | Animal Ortho 12 | | X | 4–8 | 1–2 | 2 | <1 |
| Anti-S | Human saline Fa 2-214 | S | VI | 2 | 2–4 | <1 | 1 |
| | Human incomplete Ortho | | VIII | 4 | 2 | <1 | <1 |
| Anti-P | Human Ortho | P | VI | 8–16 | 32 | <1 | <1 |
| Anti-K | Human incomplete Mor. | K | XIII | 8 | 32 | <1 | <1 |
| Anti-C | Human Ortho R3080 | C | X | 8 | 32 | <1 | <1 |
| | Human Ortho R3080 | | VI | 4 | 64 | <1 | 2 |
| | Human Ortho R3080 | | IV | 8 | 64 | <1 | <1 |
| Anti-D | Human Ortho R1208 | D | IV | 8–16 | 16 | <1 | 2 |
| | Human Ortho R1208 | | VI | 8 | 4–8 | ±1 | <1 |
| | Human Ortho R1208 | | VII | 32 | 32 | <1 | 1 |
| Anti-E | Human Ortho R4056 | E | XII | 8–16 | 16 | <1 | <1 |
| Anti-c | Human incomplete Ortho R0035 | c | VIII | 8 | 16 | <1 | <1 |
| | Human incomplete Ortho R0036 | | VIII | 8 | 8–16 | <1 | <1 |
| Anti-e | Human Ortho | e | VIII | 8–16 | 16–32 | <1 | 1 |

* Expressed as reciprocal of serum dilution.

Table 1, which shows the abolishment of agglutination of virus-treated erythrocytes by anti-M and anti-N sera. Similar results were observed when an indirect anti-human globulin test was performed on red cells "sensitized" with human anti-M sera. The absorption of all blood group agglutinins other than anti-M and anti-N by virus-treated erythrocytes, which indicates unimpaired blood group agglutinogens, can be seen in the sixth column of Table 1.

Unabsorbed anti-M and anti-N sera frequently also showed a reduction in titer when tested against virus-treated erythrocytes as compared to untreated red cells (fourth and fifth columns of Table 1). This effect was observed in spite of the interference caused by the "T" antigen, which is known to be unmasked by influenza virus and R.D.E.[4] Absorption of sera with virus-treated red cells removed "T" agglutinin.

The presence of other blood group antigens had no demonstrable effect upon the

inactivation of the erythrocyte antigens M and N by influenza viruses. Erythrocytes of different genetic origin behaved identically in this respect. Unabsorbed sera containing agglutinins against blood group antigens other than M and N sometimes gave higher, but never lower, titers with virus-treated erythrocytes when compared to intact red cells (fourth and fifth columns, Table 1). This is further evidence that none of the tested blood group antigens besides M and N is impaired by these influenza viruses.

The serological findings were essentially the same when R.D.E. was used instead of influenza viruses, in that there was inactivation of M and N receptors and no demonstrable effect on any of the other blood group antigens which were examined (Table 2).

No alteration in the blood group agglutinogens studied were observed when erythrocytes were incubated in saline or in uninoculated allantoic fluid. The activity of influenza virus and R.D.E. was destroyed by boiling.

Material released by action of influenza viruses or R.D.E. upon MM or NN erythrocytes failed to inhibit, in concentrations of 10 mg and less/ml, the action of anti-M and anti-N agglutinins upon their corresponding agglutinogens.

Color reactions performed on the enzymatically released dialyzable and nondialyzable materials were positive for carbohydrates and characteristic of neuraminic acid or its derivatives. This is in agreement with our earlier observations on digests from beef and sheep erythrocytes[1] obtained under similar experimental conditions.

## DISCUSSION

The fact that influenza viruses and R.D.E., which are known to elute virtually completely from human erythrocytes, inactivate blood group specific agglutinogens of the human red cell surface may throw light on the mode of action of influenza virus upon human cells. Under appropriate conditions, M and N receptors on the red cell surface appear to be destroyed. According to Kosjakov and Tribulev, the M and N factors occur in various human tissues besides the erythrocytes.[5] These observations have been confirmed by Boorman and Dodd.[6] Epithelial structures were found to contain more M and N active material than did tissue of mesodermal origin. Since all humans possess M or N agglutinogen or both, it seems possible that the blood group antigens M and N may serve as a point of attachment of influenza virus to human cells other than erythrocytes.

It is remarkable that no other blood group agglutinogen studied was affected by influenza virus or R.D.E. It was not possible, however, because of lack of specific antisera, to study the effect of influenza virus and R.D.E. on some rare blood group factors known to occur on human erythrocytes.

Attention should be drawn to the failure to find inactivation of the antigen S, because this antigen is considered to be the product of a gene very closely linked to the allelomorphic genes M and N.[7]

The specific removal of blood group M and N activity from the erythrocyte surface by influenza virus may point to the chemical nature of the M and N substances and the way in which these serologically active substances are bound to red cells because influenza virus is believed to possess only one enzyme, neuraminidase.[8] It is of interest in this respect, however, that Morton and Pickles[9] and Rosenfield and

Vogel[10] have described the destruction of M and N factors in addition to other blood group antigens by certain preparations of trypsin and by proteolytic enzyme preparations from *Streptomyces albus*. Watkins and Morgan[11] have observed simultaneous inactivation of blood group M and N antigens, one blood group antigen of the ABO(H) system, and influenza virus receptors by enzyme mixtures obtained from a protozoön, *Trichomonas foetus*. Similar findings have been reported by Howe, MacLennan, Mandl, and Kabat[12] with enzyme preparations from a bacterium, *Clostridium tertium*.

Blood group M and N antigens may be related chemically to the heterogenetic mononucleosis receptor of sheep and beef erythrocytes because this receptor is removed also by the agents found active against the M and N antigens.[1] Failure to observe any inhibition of anti-M and anti-N agglutinins by dialyzable and nondialyzable products from M and N erythrocytes is in contrast to our earlier observations on mononucleosis receptor, which, at least in part, was removed from erythrocytes but was not entirely destroyed by enzymatically active agents.[1] However, a more thorough study of optimal conditions should be made before concluding that the effect of influenza viruses and R.D.E. on M and N agglutinogens differs fundamentally from their action on mononucleosis receptor.

Interference caused by "T" agglutinin in those cases where the sera were not absorbed prior to testing was not always significant, especially when the original titer of the serum was high, e.g., Ortho anti-M R8064 and T2015, and the agglutinins anti-A and anti-B. In general, "T" agglutinins, even when present in high titer, were removed by one absorption with virus- or R.D.E.-treated red cells.

The titration of the specific blood group M and N activity of red cells before and after incubation with materials suspected of containing influenza virus may possibly be used to determine the presence or absence of these viruses in a given material and also of measuring antibodies to them.

SUMMARY

Influenza viruses and R.D.E. are capable of inactivating or removing blood group agglutinogens M and N from human erythrocytes. None of the other agglutinogens of human erythrocytes which were studied was demonstrably influenced by these agents. Inhibition tests performed with enzymatic digests indicated that the M and N agglutinogens are destroyed.

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* Established Ivestigator of the American Heart Association.

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## *AMINO ACID INCORPORATION IN PIGEON PANCREAS FRACTIONS**

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The function of the pancreas is predominantly that of synthesis of proteins. This tissue has therefore attracted the attention of various workers interested in this problem, for example, Mirsky,[1] Hokin,[2] Straub.[3] However, those who have attempted to obtain cell-free preparations, either for protein synthesis proper or even for an amino acid incorporation, have encountered difficulties. Yet, as a protein-synthesizing system, pancreas holds considerable promise, and for this reason we embarked some years ago on a plan to explore, if possible, the path of protein synthesis in preparations obtained from this organ.[4, 5]

At first, we also encountered difficulties in getting not only net synthesis but also amino acid incorporation, and as a preliminary we concentrated on the characterization of amino acid-activating enzymes of the type first demonstrated by Hoagland in the liver,[6] which we found rather concentrated in pancreas extracts. The trytophan-activating enzyme was particularly abundant in beef pancreas, and a fraction was obtained from particle-free extracts of this organ representing 70 per cent pure enzyme protein.[4] Studies with this enzyme have given more detailed information on the character and mechanism of action of this type of enzyme.[5]

Meanwhile, although unable to obtain in vitro enzyme synthesis in cell-free pancreas preparations, we turned, as a more hopeful approach, to amino acid incorporation. After considerable trial and error, it was found that the pancreas of young pigeons, 6–8 weeks of age, gave homogenates that consistently incorporated amino acid into protein. At first, the pancreas system appeared to be quite similar to the liver system of Zamecnik and Keller;[7] by recombining the supernate and microsome fractions, incorporation was obtained, while microsomes and supernate alone showed only slight activity. It then appeared, however, that the supernate fraction behaved differently in the pancreas from that of the liver, since reactivation could be obtained with heated supernate, while, in the liver, heating of the supernate destroyed activity. The heat-stable factor in the pancreas was then identified as a compound similar to the polynucleotide fraction of liver recently described by Hoagland, Zamecnik, and Stephenson.[8] The pancreas system therefore appeared suited to characterize this component, and it is on attempts to do this that we want to report here.

*Fractionation of Pancreas Homogenate.*—The microsomal fraction was prepared from the pancreases of 6–8-week-old pigeons by a procedure similar to that described for liver by Zamecnik and Keller,[7] for guinea-pig pancreas by Palade,[9] and for dog pancreas by Hokin.[10] The pancreas was quickly removed from pigeons killed by decapitation and homogenized in 5 volumes of ice-cold 15 per cent sucrose, in a glass homogenizer of the Bucher type.[11] Lower concentrations of sucrose give less effective fractionation, while higher concentrations of sucrose give less active particles. Nuclei, debris, and whole cells were removed by filtering through fine gauze and by centrifugation for 5 minutes at 500 *g* at 0°. The zymogen granules were removed by centrifugation at 2000 *g* for 15 minutes. At this point, three more volumes of cold 15 per cent sucrose were added, and the mitochondrial fraction was centrifuged off at 9000 *g* for 15 minutes at 0°, the high-speed head of an International centrifuge being used. The microsomal fraction was then obtained by centrifugation at 105,000 *g* for 1 hour at 0°, with the preparative Spinco Model L, and was resuspended in 8.5 per cent sucrose to a concentration of approximately 25 mg. of protein/ml. The clear 105,000 *g* supernatant or derivative preparations became the main object of our studies.

*Estimation of Protein-incorporated Radioactivity.*—After incubation, the reaction was stopped by the addition of 5 ml. of a 5 per cent solution of trichloracetic acid.[12] The precipitate was collected by centrifugation and washed three times with a similar volume of cold TCA. The residue was washed three times with 3:1 ethanol-ether and then heated at 90° for 15 minutes in 5 per cent TCA. The TCA was removed, and the residue washed once more with ethanol-ether. The white precipitate was transferred to nickel dishes, dried, and counted in a windowless gas-flow counter (Nuclear Model C-110A). The weight of each sample was accurately determined. This value was used for self-absorption corrections. The effectiveness of this washing procedure was tested by controls in which the TCA was added prior to the enzyme.

Procedures for the preparation of heated pigeon supernatant and phenol-extracted supernatant and for the counting of nucleotide-bound radioactive material are described in Figure 1 and Table 6.

The $C^{14}$-DL-amino acids used in this work were purchased from New England Nuclear Corporation. The authors wish to express their gratitude to Dr. Robert Loftfield, of the Huntington Memorial Laboratories at the Massachusetts General Hospital, for the preparation of L-leucine-1-$C^{14}$. Phosphoenolpyruvate was purchased from the California Foundation for Biochemical Research. Crystalline phosphoenolpyruvate kinase was prepared from rabbit muscle and kindly supplied by Dr. John Gregory. Lithium carbamyl phosphate and carbamyl phosphate kinase were prepared by Dr. M. E. Jones and Dr. H. Peck. A rather crude *Streptococcus faecalis* extract was free of interfering enzymes and could be used as CAP kinase. The nucleotides used in this work were the products of the Pabst Laboratories.

*The Incorporation of $C^{14}$ Leucine into Pigeon Pancreas Homogenate.*—Initially we met difficulties in obtaining incorporation, and only when turning to pigeon pancreas did results become encouraging. Even with pigeon pancreas, only with the organs of 6–8 week-old animals were consistently reproducible activities obtained. The

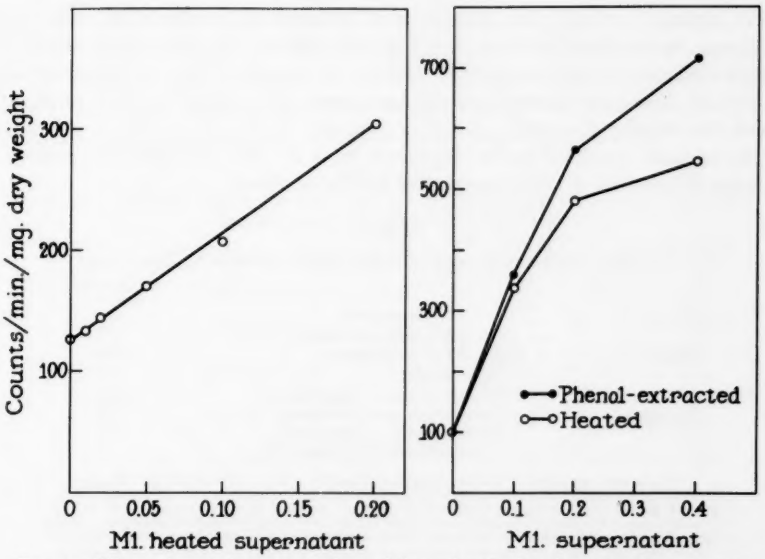


FIG. 1.—Effect of varied concentrations of heated and phenol extracted supernatant on incorporation.

Heated supernatant was prepared by placing 4 gm. of minced pigeon pancreas in 4 ml. of water, in a boiling bath for 15 minutes. A jet of hot steam was introduced to the mince at the onset of boiling, for 1 minute, to raise the temperature rapidly. The denatured preparation was cooled, the volume adjusted to 20 ml. with water, and then the tissue was homogenized with a tight-fitting glass homogenizer. Clear supernatant was obtained after spinning in the Spinco Model L at 75,000 *g* for 30 minutes. Phenol-extracted supernatant was prepared by mechanical mixing of heated supernatant with an equal volume of 90 per cent phenol (*w/v*) for 30 minutes at 0° (see n. 16). The water-phenol mixture was centrifuged at 20,000 *g* in the Servall and the water layer decanted from the phenol + precipitate. This extraction was repeated once more. The combined aqueous layers were lyophilized overnight, and the residue was suspended in cold water to half the volume of the original sample. Slight traces of phenol remained.

The heated supernatant was lyophilized and resuspended in half the original volume in water. The conditions for the enzyme incubation were identical with those of Table 1. Each tube contained approximately 4.2 mg. of microsomal protein.

first task now was to test activities in various fractions or combinations thereof. In Table 1, a survey of such experiments is presented. It shows that, as in liver,[7] incorporation into protein depends on a combination of microsome and supernatant fraction. It is shown in Tables 1 and 2 that, without an energy source, i.e., a phosphate bond feeder +ATP, no incorporation whatsoever is obtained. With supernatant alone, no incorporation into protein was seen, but microsomes retained about one-third to one-fourth of the maximum activity elicited on addition of supernatant. The addition of 0.2 $\mu g$. of crystalline pancreatic ribonuclease completely inhibited the activity of the combined fractions.

The highest incorporation of leucine took place in the pH range 8.0–8.2. The activity of the system fell off rapidly at pH values below 7.8 and above 8.4.

The enzymatic incorporation of leucine into microsomal protein required the presence of magnesium. Optimal incorporation occurred when the magnesium ion concentration was $0.005M$. Manganese or calcium at equivalent concentrations were unable to activate the system.

For optimal activity, the system also required the presence of added salts. KCl was more effective than NaCl in stimulating leucine incorporation. At concentrations of NaCl higher than 0.05 $M$, the activity fell. At a concentration of 0.05 $M$, KCl gave optimal activity and remained constant up to 0.10 $M$, after which the activity dropped.

The system appeared to be saturated at $5 \times 10^{-5}$ $M$ L-leucine, and in 15 minutes 80 per cent of the incorporation had taken place.

TABLE 1*

INCORPORATION OF LEUCINE-1-$C^{14}$ INTO PIGEON PANCREAS FRACTIONS

| Fractions | Homogenate† | c.p.m./Mg. Dry Wt. |
|---|---|---|
| | | 472 |
| Single | 9,000 $g$ (mitochondria) | 75 |
| | 105,000 $g$ (microsomes) | 160 |
| | Supernatant | 20 |
| Combined | Mitochondria + supernatant | 241 |
| | Microsomes + supernatant | 593 |
| | Microsomes + supernatant but without ATP and CAP | 9 |

* Each tube contained 5 $\mu$moles of $MgCl_2$; 50 $\mu$moles of Tris buffer of pH 8.1; 50 $\mu$moles of KCl; 0.1 $\mu$moles of DL-leucine-$C^{14}$ ($7.5 \times 10^6$ c.p.m. per micromole); 10 $\mu$moles CAP, CAP kinase (crude extract of *Streptococcus faecalis*); 1 $\mu$mole of ATP; 0.4 $\mu$mole of GTP; and supernatant derived from approximately 0.2 gm. wet weight of pigeon pancreas. The final volume of the system was 1.0 ml. The tubes were incubated for 30 minutes at 37°.

† After removal of cell debris, nuclei, and zymogen granules by 2,000 $g$ centrifugation.

TABLE 2*

EFFECT OF VARIOUS SUPERNATANT FRACTIONS ON LEUCINE INCORPORATION INTO PIGEON PANCREAS MICROSOMES

| Additions | c.p.m./Mg. Dry Wt. |
|---|---|
| None | 182 |
| Whole supernatant | 488 |
| pH 5 precipitate | 147 |
| pH 5 supernatant | 325 |
| Heated† whole supernatant | 470 |
| Heated whole supernatant but without ATP and CAP | 10 |

* To the 100,000 $\times$ $g$ supernatant (whole supernatant) from pigeon pancreas, molar acetic acid was added in small portions at 0° until pH 5 was reached. The pH 5 precipitate and supernatant were collected by centrifugation and neutralized with solid $KHCO_3$. The volume of the pH 5 precipitate was readjusted to the initial volume. The conditions for incubation were identical with those of Table 1. Each tube contained approximately 4.0 mg. of microsomal protein.

† Kept for 15 minutes in boiling water.

It soon appeared, however, that the similarity to the liver system was not complete. As elaborated by Keller and Zamecnik and their collaborators[13] and as already concluded earlier by Siekevitz in Zamecnik's laboratory,[14] the activity which complements the microsomes is precipitated at pH 5. This pH 5 fraction, on further analysis, was found by Hoagland[6] and by Hoagland, Keller, and Zamecnik[15] to contain the amino acid-activating enzymes. The data of Table 2 illustrate the differences between pancreas and liver. The pH 5 precipitate of pancreas supernate did not complement the microsomes, while the pH 5 supernate did. Furthermore, as shown in No. 5 in Table 2, activity could be restored to the microsomes with boiled supernatant, although the reaction mixture contained excess GTP, which

Keller and Zamecnik[13] had found to be a component of the liver system. This effect of the boiled supernate was obtained not only with leucine but, as may be seen in Table 3, with a number of other amino acids also, and therefore appears to be of a general nature. It is with the preliminary identification of this heat-stable component that we shall be dealing mostly from now on.

TABLE 3*

STIMULATION BY BOILED SUPERNATANT ON INCORPORATION OF VARIOUS LABELED AMINO ACIDS INTO MICROSOMAL PROTEIN

| Experiment No. | Labeled Amino Acid | Boiled Supernatant | c.p.m./Mg. Dry Wt.† |
|---|---|---|---|
| 1 | DL-Leucine-1-$C^{14}$ | − | 98 |
| | | + | 357 |
| 2 | DL-Serine-3-$C^{14}$ | − | 120 |
| | | + | 341 |
| 3 | DL-Valine-1-$C^{14}$ | − | 91 |
| | | + | 379 |
| 4 | DL-Alanine-1-$C^{14}$ | − | 133 |
| | | + | 221 |
| 5 | DL-Phenylalanine-3-$C^{14}$ | − | 132 |
| | | + | 158 |

* The conditions for incubation were identical with those shown in Table 1 except for the concentrations of amino acid, which were as follows: 0.1 μmole DL-leucine-1-$C^{14}$ ($7.5 \times 10^6$ c.p.m. per micromole); 0.4 μmole DL-serine-3-$C^{14}$ ($3.1 \times 10^6$ c.p.m. per micromole); 0.4 μmole DL-valine-1-$C^{14}$ ($2.0 \times 10^6$ c.p.m. per micromole); 0.4 μmole DL-alanine-1-$C^{14}$ ($3.7 \times 10^6$ c.p.m. per micromole); and DL-phenylalanine-3-$C^{14}$ ($2.3 \times 10^6$ c.p.m. per micromole). Each tube contained approximately 4.3 mg. of microsomal protein as determined by biuret reagent; 0.40 ml. of boiled supernatant was used as prepared from a 20 per cent pigeon pancreas homogenate.

† For better comparison, the specific activities of the other amino acids were adjusted to that of leucine.

As shown in Figure 1, the heated supernate factor can be assayed with the microsome fraction. This activity of heated supernate was, at first, rather puzzling. It was found that the pancreas microsomes retained amino acid-activating enzymes in reasonably large amounts when assayed by pyrophosphate exchange. Even after washing twice, a considerable pyrophosphate exchange was found with amino acid mixture or leucine. As shown in Tables 4 and 5, however, with dilution or washing, the activation by the heated supernate is lost, and now incorporation requires unheated supernate. In view of the persistence of amino acid-dependent ATP-pyrophosphate exchange in the washed microsomes, the enzymatic factor washed out most easily may be an enzyme functioning in another phase of amino acid transfer. We have not yet attempted to characterize this function more closely.

*Preliminary Characterization of the Heat-stable Complement.*—The material remaining soluble on heating is predominantly of a polynucleotide nature. Unfortunately, no good source for this material has been found so far, except the pancreas of young pigeons. Liver also contains this complement; however, it is closely associated there with protein and is obtained protein-free only after phenol treatment.[8]

If heating of the supernatant is carried out slowly, large losses of activity occur. Also, on dialyzing unheated supernate, almost all activity is lost, while, after heating, the complement proved to be non-dialyzable. Most likely, in these cases, the factor is enzymatically inactivated, presumably by ribonuclease. We also believe enzymatic inactivation to be the cause for the inactivity we found with extracts, of slaughterhouse-obtained pancreas.

As mentioned, the activator is non-dialyzable. At first, there were difficulties in showing the non-dialyzability because dialysis seemed to destroy activity. However, after the cellophane tubes had been washed with versene, inactivation disappeared, and after dialysis all the activity was found inside the cellophane bag. When subjected to electrophoresis on the Geon 426 resin (Goodrich), the active material migrates rather fast. The peak of activity, illustrated by Figure 2, largely coincides with the peak of absorption at 260. Ultracentrifugation of the peak fraction of Figure 2, kindly carried out by Miss Mary Peterson, of the Sloan Kettering Institute, showed a broad peak corresponding to molecular weights between 20,000 and 40,000 and indicated gross inhomogeneity.

TABLE 4*

INCORPORATION INTO MICROSOMES SEDIMENTED FROM DILUTED PANCREAS HOMOGENATES

| HOMOGENATE DILUTION FROM WHICH MICROSOMES PREPARED | C.P.M./MG DRY WT. | | |
|---|---|---|---|
| | No Addition | Supernatant | Heated Supernatant |
| Routine | 144 | 352 | 246 |
| 4× | 105 | 392 | 127 |
| 6× | 42 | 437 | 78 |

* The routine microsome preparation as described is fourfold diluted with regard to the initial pancreas homogenate. The 4× and 6× dilutions of the routine fraction were prepared by the addition of appropriate volumes of cold 15 per cent sucrose just prior to the sedimentation of particles at 105,000 *g*. The conditions for the enzyme incubation were identical with those shown in Table 1. Microsomal protein ranged between 4.0 and 4.5 mg. per tube for the various preparations; 0.4 ml. of the heated and unheated supernatant was used as prepared from a 20 per cent pigeon pancreas homogenate.

TABLE 5*

LOSS OF INCORPORATION BY WASHING OF MICROSOMES

| | C.P.M./MG. DRY WT. | | |
|---|---|---|---|
| | No Addition | Supernatant | Heated Supernatant |
| Microsomes | 57 | 231 | 116 |
| Microsomes, washed | 37 | 179 | 37 |

* Washed microsomes were prepared by resuspending the 105,000 *g* sediment in 2 volumes of 8.5 per cent sucrose and resedimentation in the Spinco at 105,000 *g*. The conditions for the enzyme incubation were identical with those shown in Table 1, except that each tube contained 15 μmoles of CAP. Microsomal protein for each tube ranged between 3.4 and 3.8 mg; 0.4 ml. of heated and unheated supernatant was used, as prepared from a 20 per cent pigeon pancreas homogenate.

So far, the experiments reported indicate that a polynucleotide acted as a mediator between the amino acid-activating enzyme and the microsome. If this were so, then in the absence of the microsomes the polynucleotide should be charged with amino acid. To test for this, a preparation of the supernate was used which, through dialysis, was depleted of carrier. To this supernatant, containing the activating enzymatic factors, a concentrated preparation of the polynucleotide was added which contained about ten to fifteen times as much polynucleotide as was ordinarily used in the previous experiments. Such an experiment is presented in Table 6.

Two methods for collecting the polynucleotide and washing were used. By acid ethanol precipitation,[18] the polynucleotide as well as protein was collected. It is significant that counts disappear when this precipitate is treated with hot TCA,[19] which indicates that the labeled leucine is not incorporated into protein, in which case hot TCA would not have removed the counts. On the other hand, the ex-

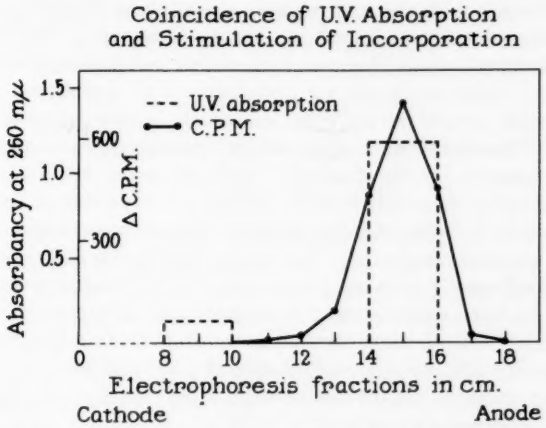


FIG. 2.—Coincidence of UV absorption and stimulation of incorporation. Concentrated phenol-extracted supernatant, prepared as described in Fig. 1, was dialyzed and electrophoresed on Geon 426 Resin for 19 hours at 150 volts in 0.1 molar tris buffer of pH 8.1 at 0°. After electrophoresis, the resin was cut into 1-cm. segments, and each fraction was eluted with water. Based on absorbancy at 260 millimicrons in the Beckman DU Spectrophotometer, 86 per cent of the material placed on the resin was recovered. Fractions 8–10 and 14–16 were combined and tested for activity toward the stimulation of incorporation into protein in the assay system. The conditions for the enzyme assay were identical to those of Table 1. The delta c.p.m. in this figure represent the total counts incorporated in the presence of the above combined fractions minus the counts incorporated by the microsomes alone.

TABLE 6*

TRANSFER OF CARBOXYL-LABELED LEUCINE TO POLYNUCLEOTIDE "FACTOR"

| Incubation Mixture | A Washed Acid-Alcohol Ppt. | B Phenol-Extracted Non-dialyzable |
|---|---|---|
| | Total Counts/Min | |
| Boiled supernatant + "factor" | 92 | 103 |
| Supernatant, 0.25 ml. alone | 49 | 43 |
| Supernatant, 0.25 ml. + 0.4 ml. "factor" (= 5 ml. super.†) | 2060 | 1993 |
| Same | 26 (hot TCA wash) | ... |

* Twenty per cent homogenate of pigeon pancreas in 15 per cent sucrose was centrifuged at 100,000 *g* for 1 hour in the Spinco Model L, and the clear supernatant was collected and dialyzed overnight against distilled water. Phenol-extracted supernatant concentrate, prepared as described in Fig. 1, was used as acceptor. 50 μmoles of tris buffer of pH 7.4; 5.0 μmoles of $MgCl_2$; 0.25 μmole of DL-leucine-1-$C^{14}$; 2.0 μmoles of ATP; 10 μmoles of CAP. CAP kinase; 0.25 ml. supernatant enzyme; and 0.40 ml. of factor (= 5 ml. supernatant) were incubated in a total volume of 1.0 ml. for 10 minutes at 37°. At the end of the reaction, phenol-extracted factor was added to those tubes which did not contain the material, and 2 mg. carrier yeast RNA was added to all vessels.

Set A: In one set of tubes, the reaction was stopped by the addition of a cold acid-ethanol reagent (935 ml. ethanol, 60 ml. glacial acetic acid, and 4.5 ml. of 2 *N* NaOH) and allowed to stand for 1 hour at 0° (see n. 17). The precipitate was centrifuged and washed five times with this reagent. The precipitate was then washed twice with cold 5 per cent TCA. Tube No. 4 was, in addition, extracted with TCA for 15 minutes at 90°. Finally, the residue was washed with ethanol-ether and counted for radioactivity.

Set B: In another set of tubes, 90 per cent phenol was added after incubation and extracted for 45 minutes at room temperature. The mixture was centrifuged at 10,000 *g*, and the clear top aqueous layer was removed by capillary pipette. The remaining phenol was extracted twice more with an equal volume of water. The aqueous portions were combined and subjected to exhaustive dialysis against water until no further counts could be detected in the dialyzate. Aliquots from each dialysis tubing were counted for radioactivity.

† Concentrate after phenol extraction.

traction of the same material with phenol, according to Kirby[16] and Gierer and Schramm,[17] more directly identified the counts as bound to the polynucleotide material of the same type as identified earlier with liver preparation by Hoagland *et al.*[8] as a factor in the amino acid incorporation. This experiment indicates that the polynucleotide accepts the activated amino acid in some manner.

*Comments.*—The experiments with cell-free preparations of pigeon pancreas amplify the experience of the Zamecnik-Hoagland group with liver preparation. It appears that, after the amino acid is activated, it is transferred to a polynucleotide carrier before it is eventually deposited in the microsome. This transfer presents a rather complex picture. The sequence of events is not quite clear yet, and a thorough discussion is better postponed until more details are available.

*Summary.*—In the study of cell-free preparations of pigeon pancreas, amino acid incorporation into microsome protein is shown to occur if a probably complex mixture of enzyme proteins and one or several polynucleotides is added. These polynucleotides are present in the non-particulate fraction of pigeon pancreas. They are heat-stable and non-dialyzable.

Amino acid transfer to the polynucleotide carrier can be shown on incubation with dialyzed supernate. It is concluded that this polynucleotide component acts as an intermediary between activating enzymes and microsomes, cf. also.[3]

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# *A PROBABLE MECHANISM OF ACTION OF SEROTONIN*

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Although the hormone serotonin (5-hydroxytryptamine) is known principally because it causes smooth muscles to contract, the reason for this action is quite unknown. The purpose of this paper is to present evidence for the view that serotonin exerts its physiological effects by carrying calcium ions across the membrane into animal cells.

It is now well established that the isolated uterus of an estrogenized rat is a desirable test object in which to observe the muscle-contracting action of serotonin.[1–3] Very small amounts of the hormone (0.003–0.01 $\mu$g/cc.) cause this isolated tissue to contract strongly. We have observed that calcium ions or potassium ions will also make this tissue respond in the way it does to serotonin. The ions, however, are far less potent than the hormone. Under the conditions to be described below, calcium chloride was 70,000 times less potent (on a molar basis) than serotonin. Potassium chloride was only one-fourth as potent as calcium chloride. Magnesium chloride was inactive, although, when it had been present in the bath in high concentration, repeated contractions of the muscle always occurred after washing. The first point of evidence, therefore, which linked serotonin to these ions was that calcium ions (and to a lesser extent potassium ions) had the qualitative activity of serotonin on the muscle.

The activity of calcium ions was discovered during the assay of tissue extracts for serotonin. Some of these extracts contained considerable quantities of calcium ions. When control solutions containing only these ions but no other components of tissue were assayed, the serotonin-like activity was found.

The second point of evidence which suggested that metallic ions were concerned in the action of serotonin was that, when an isolated uterus was treated with versene (ethylenediaminetetraacetate), serotonin no longer was able to cause a contraction. When the versene was washed away or neutralized by calcium ions, serotonin again caused the muscle to contract. This demonstrated that the versene had not brought about irreversible damage to the tissue.

These experiments were done in the following way. Virgin female rats were estrogenized with a single intraperitoneal injection of 0.04 mg. of diethylstilbestrol. Twenty hours later the animals were killed, and a horn of the uterus was removed and suspended in oxygenated salt solution of the following composition: NaCl, 9 gm.; KCl, 420 mg.; $CaCl_2$, 20 mg.; $NaHCO_3$, 0.5 gm.; glucose, 0.5 gm.; water, 1 liter. One end of the tissue was attached to the lever of a kymograph. The preparation was washed by rapid emptying of the bath and prompt refilling. Temperature was 30° C. Additions of test compounds were made in small volume to the saline solution while it was in contact with the tissue.

The record shown in Figure 1 indicates that, qualitatively, the contractions caused by serotonin and by calcium chloride were similar but that there were signifi-

cant differences. When an amount of serotonin was used which was just sufficient to cause maximal contraction, a series of responses resulted in which each succeeding contraction was less than the preceding one. With $CaCl_2$, however, the diminution did not occur. The diminution of response with serotonin was probably due to some destruction of the hormone during each contraction because it could be circumvented by small additions of serotonin (one-fifth of the original exciting dose) after each contraction. This qualitative difference in effect of the hormone and of $CaCl_2$ could thus be eliminated. A second qualitative difference, however, remained. This was that the lag between the addition of the contractant and the response of the muscle was always longer with calcium chloride than with serotonin. Nevertheless, with either substance a series of contractions occurred. Figure 1 also shows that, when versene was applied, a single contraction was elicited, but the repeated contractions seen with serotonin or with calcium chloride were not observed. The reason for the single contraction caused by versene is not well understood.

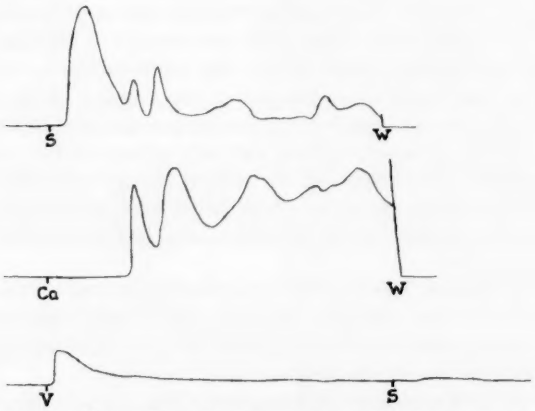


FIG. 1.—Comparison of responses of an isolated rat uterus to serotonin, calcium chloride, and versene. *S* = serotonin 0.1 $\mu g$/10 cc; *W* = wash; *Ca* = $CaCl_2$, 2 mg/10 cc; *V* = versene, 1 mg/10 cc.

When the tissue was in the presence of versene, serotonin would not evoke a contraction (Figs. 1 and 2). However, when the versene was removed by repeated washings or neutralized by calcium ions (Fig. 2), serotonin again brought about the usual response. Versene also blocked the response of the muscle to potassium even though the sequestering of this ion is usually considered to be difficult.

In addition to serotonin, acetylcholine is also able to cause contractions of the isolated rat uterus. This ability of acetylcholine was also blocked by versene. However, repeated washing of the tissue did not restore its ability to respond to acetylcholine, as had been the case in the serotonin experiments. Instead, the ability to respond seemed permanently impaired. If serotonin was applied to such a tissue, a usual contraction occurred. When this response had been obtained, washing and reapplication of acetylcholine then resulted in contraction.[4]

The above phenomena can be understood in the following terms. Szent-Györgyi[5] and Csapo[6] have pointed out that an isolated thread of actomyosin in the presence of adenosine triphosphate can be made to contract only with suitable metallic ions ($Ca^{++}$ or $Mg^{++}$). The hormones which are known to cause muscular contraction, such as epinephrine, acetylcholine, or oxytocin, will not cause these isolated threads to contract. To this list of hormones we may also add serotonin. Clearly, in the intact muscle there is an apparatus which is activated by such hormones, but this apparatus is not functioning in the threads of actomyosin. Csapo has suggested that this apparatus is possibly in the cell membrane.

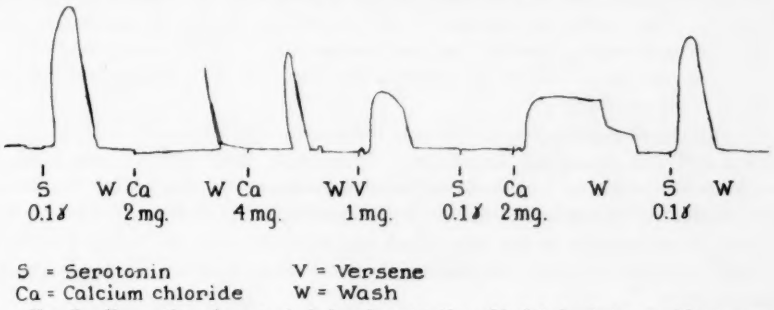


FIG. 2.—Prevention of serotonin-induced contraction of isolated rat uterus with versene and reversal of this effect. Amounts shown are per 10 cc.

To understand the present experimental findings about serotonin, we may make the following first approximation to the truth. Calcium ions diffuse through the cell membrane of the estrogenized rat uterus, but only with considerable difficulty. If the concentration outside is high enough (0.22 mg $CaCl_2$/cc in the present experiments), enough calcium ions diffuse into the cell to result in a contraction when they reach the ATP and actomyosin. The lag indicated in the figures may be related to the time required for this diffusion. Normally, however, when there are only 0.02 mg $CaCl_2$/cc, there are not enough calcium ions to penetrate and cause a contraction, and the muscle remains quiet. If one accepts the view (for which there is much experimental evidence) that the cell membrane is principally lipoidal with a network of proteinaceous or polysaccharide-like material dispersed in it, the difficulty in getting calcium ions across such a membrane can be appreciated. These ions are not soluble in lipid. Perhaps the only way for them to penetrate the membrane by simple diffusion is to pass through the non-lipoidal network. Since this network constitutes only a fraction of the membrane, it might be expected that enough calcium ions could enter the cell by this means only if the concentration outside were raised considerably. In the present experiments the increase in calcium ion concentration required was tenfold.

We may picture a substance in the lipoidal part of the membrane which combines both with serotonin and with calcium ions (but not with just one of them). The compound so formed is fat-soluble. When serotonin is added to a uterus suspended in the saline solution of relatively low calcium content, this compound forms. It then diffuses across the cell membrane. On the inside of this membrane it encounters an enzyme which destroys it, with the consequent liberation of calcium

ions inside the cell. If we think of this portering substance (as Vogt[7] and Bennet-Clark[8] have done in related situations with respect to other ions) as a phospholipid, then the removal of a phosphate or choline grouping would be all that is required to set the calcium ions free. The slightly degraded portering substance could then diffuse back across the lipid portion of the cell membrane and could be regenerated by an enzyme system which would restore the phosphate or choline grouping to it. It would then be ready to combine with more serotonin plus calcium ions and repeat the transport process. It is, of course, possible that this portering substance is not a phosphatide but rather some other specialized lipoidal constituent.

Once the calcium ions have entered the cell, a mechanism is required to get them out again. The trading of positions of the two enzyme systems postulated above, so that the synthesizing system is on the interior side and the hydrolyzing one on the exterior, can be pictured to account for the return of the calcium, and possibly of some[9] of the serotonin.

The analysis of muscles for calcium ions before and after treatment with serotonin is not a sufficient test of this hypothesis. Many such analyses have been made in muscles before and after a contraction induced electrically. The failure to observe large changes in the calcium content is believed by some to be explained by the presence of compounds in the cells which can combine with and either release or sequester calcium ions, thus beclouding the issue when analyses are made on disrupted cells.

Quite obviously, many details of the proposed mechanism remain to be elucidated. One advantage, however, is that the present scheme accommodates the existing data and, more importantly, that it can be tested directly by experimentation. For example, it should be possible to isolate the postulated portering substance. For the present we wish to point out only that the action of serotonin on smooth muscles is closely related to the effects of calcium ions. The main facts can be understood in terms of the formation of a compound from a membrane component, serotonin, and calcium ions, for the transport of calcium ions into the cell. If this view should prove to be correct, then the mechanism of action of the hormone serotonin would be to activate a special "valve" for admission of calcium ions into the cell. The serotonin receptor would then be the postulated membrane substance and its associated two enzyme systems.

Perhaps there are other "valves" actuated by the other hormones for the admission of these same metallic ions. Thus, if histamine or oxytocin each opens a different "valve" for the admission of, let us say, calcium ions, it becomes understandable why each of these hormones causes contraction of the muscle, but only the metallic ions cause the isolated threads of actomyosin to shorten.

The present concept of the mode of action of serotonin may have a relationship to the understanding of how indoleacetic acid acts on plant cells. Recently evidence has been presented which indicates that serotonin in animals is the analogous hormone to indoleacetic acid in plants.[10] It is therefore of interest that Bennet-Clark[8] in 1956 published a hypothesis for the mode of action of indoleacetic acid which resembles in some respects the one now proposed for serotonin. He did not specifically implicate calcium ion transport but spoke more generally of ion transport. He envisioned lecithin as the portering substance and pictured it as being degraded by loss of choline and resynthesized through the action of choline esterase.

Although his scheme differed in detail from the present one, there is nothing to say that either his scheme for plants or the present one for animals should be regarded as final. Both are admittedly working hypotheses and can only be regarded as first approximations to the truth. Bennet-Clark's idea of cleavage of choline from the phosphatide by synthesis of acetylcholine and the resynthesis of such a phosphatide by reversal of such a reaction might well help to explain the curious dependence of acetylcholine response in versene-treated uterus on prior treatment with serotonin, a phenomenon noted in the present work.

* With the technical assistance of N. K. Campbell and M. Gallagher. This paper was presented orally at the fall meeting of the National Academy of Sciences, November 18, 1957, and abstracted in *Science*, **126**, 1236, 1957.

[1] V. Erspamer, *Arch. internat. pharmacodynamie*, **103**, 293, 1953.

[2] J. H. Gaddum and K. A. Hameed, *Brit. J. Pharmacol.*, **9**, 240, 1954.

[3] E. Shaw and D. W. Woolley, *J. Pharmacol. Exptl. Therap.*, **111**, 43, 1953.

[4] This dependence of the acetylcholine response in versene-treated muscle on the prior treatment with serotonin may prove useful as a specific pharmacological test for serotonin or for acetylcholine. In any event, it illustrates the interplay of these two hormones, a fact which is becoming increasingly clear from a variety of pharmacological experiments.

[5] A. Szent-Györgyi, *Chemical Physiology of Contraction in Body and Heart Muscle* (New York: Academic Press, 1953).

[6] A. Csapo, *Proceedings of the 1955 Laurentian Hormone Conference* (New York: Academic Press, 1956), **12**, 405.

[7] W. Vogt, *Nature*, **179**, 300, 1957.

[8] T. A. Bennet-Clark, in *The Chemistry and Mode of Action of Plant Growth Substances* (London: Butterworth's Scientific Publications, 1956), p. 284.

[9] The experiments cited earlier in this paper indicated that a fraction of the serotonin was lost during each contraction.

[10] D. W. Woolley, *Nature*, **180**, 630, 1957.

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## *EXTREMAL PRINCIPLES FOR SIMULATING TRAFFIC FLOW IN A NETWORK**

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*Introduction.*—Rational long-range planning of city traffic networks has focused attention on the desirability of research directed toward an expedient method for developing and analyzing traffic loads and requirements from origins to destinations over city streets. To aid in solving this kind of problem, W. Prager[1] formulated a mathematical model of traffic-network flows which would, by means of an external principle, imply satisfaction of one of two principles enunciated by J. G. Wardrop,[2] viz., all alternate routes which are actually used by vehicles traveling between two points require the same journey time.

Although Prager achieved an analytic formulation (which is analogous to the association of electrical potentials at the nodes of a direct-current circuit) his approach leaves problems such as the following to be dealt with: (1) on each branch

of the network the flow is required to be mainly in one direction (called "main-stream" as contrasted with "cross-stream"); (2) these directions must be specified in advance; (3) interchanges between main- and cross-streams are not permitted at any intersection; (4) only origin effluxes, destination influxes, and conservation of flow at intersections may be prescribed; and (5) a convex quadratic frictional dissipation law is assumed for each branch.

Two types of extremal principles will now be presented which (*a*) allow opposing directions of flow on a branch to be of any relative magnitude, (*b*) eliminate the need for specifying main- and cross-stream directions, (*c*) permit interchanges which are consistent with the network, (*d*) insure that all origin-to-destination requirements will be met, and (*e*) via the construction of R. J. Duffin[3] permit arbitrary convex and anisotropic branch dissipation laws.

*Polyextremal Principles for a Single Road Network.*—Traffic patterns formed from "emergent (repetitive) steady-state" conditions can be viewed as resulting from a "shaking-down" (toward equilibrium) which occurs through the coursing of many vehicles over the network in pursuit of their objectives.[4] This suggests a game-theory analogy.[10] A player is associated with each origin who seeks to distribute his vehicles to various destinations in required amounts.[5] Each such player strives to fulfill his flow requirements while operating in a network allowing two-way flow on each branch with resistance characteristics—e.g., time of flow over branches—which are partly determined by the flow actions of other players.

By Duffin's[3] branch-resistance to extremal potential-principle, each player in this (multiperson) game may be viewed as striving to minimize the sum of the integrals of his branch-current resistances. Then satisfaction of all origin-to-destination requirements, as well as conformity (for the totality of traffic flow) to a "Wardrop-like" principle, is guaranteed by requiring polyextremal equilibrium, e.g., a "Nash equilibrium" or its "$k$-stable" variants which exist (by the Nash[6] arguments) under conditions cited above. Many computational devices are available, such as loadings from the origins in sequence, which are alternated until convergence is secured; also, randomization or other suitable devices may be used to secure a unique equilibrium.[7]

*Multicopy Network Models.*—The previous designs may be comprehended within a single extremization which provides access to the evaluative and computational efficiencies of mathematical (and even linear) programming.[8] Associate a copy of the network with the $\alpha$th player. Let $x_j^\alpha$ be the current in the $j$th branch of this copy. Then

$$\sum \epsilon_{ij}{}^\alpha x_j{}^\alpha = E_i{}^\alpha, \tag{1}$$

where

$\epsilon_{ij}{}^\alpha$ = the incidence numbers[1] (which may be chosen differently for each copy),

$E_i{}^\alpha$ = the effluxes or influxes associated with the $\alpha$th origin-to-destination requirements,

are the Kirchoff node conditions for the $\alpha$th copy.

The integrated resistance function, $R_j$, of the actual $j$th branch of the network may be written $R_j(\sum|x_j{}^\alpha|)$.

The principle is, then,

$$\text{Min.} \sum R_j \left(\sum_\alpha |x_j^\alpha|\right),$$

subject to

$$\sum_j \epsilon_{ij}^\alpha x_j^\alpha = E_i^\alpha. \tag{2.1}$$

or, introducing Lagrange multipliers, $\lambda_i^\alpha$, figuratively,

$$\text{Min.} \sum_j R_j \left(\sum_\alpha |x_j^\alpha|\right) + \sum_\alpha \sum_i \lambda_i^\alpha \left(\sum \epsilon_{ij}^\alpha x_j^\alpha - E_i^\alpha\right). \tag{2.2}$$

The optimal $\lambda_i^\alpha$ play the role of node potentials in the $\alpha$th copy. This principle can be generalized to allow inequality (e.g., policy) conditions on the $x_j^\alpha$ as well as anisotropic forms for the integrated resistance functions.

When the $R_j$ are piecewise linear (e.g., via an approximation), the principle can be written in the form

$$\text{Min.} \sum_\alpha \sum_j R_j |x_j^\alpha|,$$

subject to

$$\sum_j \epsilon_{ij}^\alpha x_j^\alpha = E_i^\alpha, \tag{3.1}$$

$$\sum_\alpha |x_j^\alpha| \leq \Delta_j,$$

corresponding to replacement of the original $j$th branch by several new ones, each with a definite capacity and a different integrated resistance law which is linear in the total traffic on this branch. An equivalent linear programming formulation is

$$\text{Min.} \sum_j R_j \times (x_{j+}^\alpha + x_{j-}^\alpha),$$

subject to

$$\sum_j \epsilon_{ij}^\alpha (x_{j+}^\alpha - x_{j-}^\alpha) = E_i^\alpha, \tag{3.2}$$

$$-\sum_\alpha (x_{j+}^\alpha + x_{j-}^\alpha) \geq -\Delta_j,$$

$$x_{j+}^\alpha, x_{j-}^\alpha \geq 0.$$

Its dual is

$$\text{Max.} \sum_\alpha \sum_i \varphi_i^\alpha E_i^\alpha - \sum_j \psi_j \Delta_j.$$

subject to

$$\sum_\alpha \varphi_i^\alpha \epsilon_{ij}^\alpha \leq R_j + \psi_j, \tag{3.3}$$

$$\psi_j \geq 0.$$

Optimal solutions $x_j^{\alpha *}$ and $\varphi_i^{\alpha *}$, $\psi_j^*$ satisfy

$$\sum_j R_j \, sgn \, (x_j^{\alpha *}) x_j^{\alpha *} = \sum_\alpha \sum_i \varphi_i^{\alpha *} E_i^\alpha - \sum_j \psi_j^* \Delta_j. \tag{4}$$

Thus evaluators are provided for the rates of change in $E_i^\alpha$ and $\Delta_j$ with extremal simulation of the traffic pattern.

The special network form and the sole interconnection of copies through the "$\Delta_j$ conditions" invite special attention to efficient methods of computation. Also, formulae (2) and (3) suggest electric analogue computers.

The multicopy model can be viewed as an instance of a dynamic network in which the $\alpha$th copy corresponds to conditions in the $\alpha$th period and the $\Delta_j$ conditions provide interperiod connections. Finally, the multicopy programming model may be regarded as defining a new type of solution for the class of multiperson games involved in the polyextremal principle. In particular, when $R_j$ is strictly convex, a unique Nash equilibrium is selected.

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[4] Rush-hour traffic and average daily flows are examples often cited as fulfilling these conditions.

[5] Additional ("artificial" or "phony") origins may be employed when different types of vehicles (which congest) need to be considered.

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# *EARLY UNSTABLE $CO_2$ FIXATION PRODUCTS IN PHOTOSYNTHESIS**

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A comparison of the influence of different killing procedures on $CO_2$ fixation in short periods of photosynthesis (5–10 seconds) suggested that a part of the $CO_2$ is unstably bound.[1] Cold solvents extract some of this labile complex, but it apparently decomposes during the following fractionation and counting procedure. If the algae are poured into boiling ethanol, however, less $CO_2$ appears to be fixed, presumably because the unstable compound(s) is at least partly decomposed to give back $CO_2$.[2] The addition of hydroxylamine (to a concentration of 0.1 *M*) to the algal suspension immediately before the killing process gives a significantly higher $CO_2$ fixation than occurs in the absence of hydroxylamine. The addtional fixation product(s) is relatively stable during the succeeding procedure.

To study this phenomenon in greater detail, *Scenedesmus* suspensions were allowed to assimilate $C^{14}O_2$ for periods between 5 seconds and 5 minutes. They were then killed by acetone or ethanol at about −30° C. After the addition of acetic acid (to remove excess radioactive bicarbonate) an aliquot was sealed (after rapid evacuation at −45° C.) into a glass bomb containing a copper–copper oxide mixture together with some glucose as a carrier substance.[3] Most of the manipulation and storage of these bombs was carried out in a liquid oxygen bath, and the temperature was not allowed to rise above −20° C. at any time before the combustion vessel was sealed. The organic material was burned (at about 650° C.), and the activity of the resulting $CO_2$ was measured in an ionization chamber with a vibrating reed electrometer. By this means a value for the "total fixation" was obtained. The difference between this amount and that obtained by the standard procedure of plating the suspension on aluminum planchets and counting with a Geiger tube gives the amount of "unstable fixation." It was found that after 5 seconds of photosynthesis more than half the activity was bound in a labile complex which was lost during the normal procedure. At least some of this loss can be prevented by the addition of hydroxylamine to the suspension either before, together with, or after (if the temperature is low) the solvent used for the killing of the algae is added. Thus the experiments with the gas-counting method confirmed the results of our earlier experiments, in which the stabilizing effect of the hydroxylamine was indicated.[2] Table 1 gives some data obtained by the usual plate-counting procedure which illustrate the effect of both alcohol temperature and hydroxylamine on the amount of $CO_2$ fixed. The influence of the hydroxylamine upon the fraction of the activity which is soluble in ethanol is not very pronounced. In all acetone experiments, however, a marked effect of hydroxylamine on the activity distribution between soluble and insoluble components is apparent (Table 2). At least a part of the increased fraction of the activity appearing in the acetone-soluble components is accounted for by the increased amount of stable phosphate esters apparently brought into solution by the hydroxylamine. It appears as if the hydroxyl-

amine liberated the phosphate from either a salt or an anhydride-type linkage with the enzyme, thus allowing the phosphate to dissolve. The application of hydroxylamine may very well lead to the detection of new phosphorylated compounds which are retained by the insoluble fraction during the normal extraction procedure.

TABLE 1

INFLUENCE OF $NH_2OH$ ON THE STABLE $CO_2$ FIXATION*

| Killing Procedure | Without $NH_2OH$ | With $NH_2OH$ |
|---|---|---|
| Boiling ethanol | 3,098 | 5,892 |
| Ethanol (−35° C.) | 5,506 | 7,390 |

5-second photosynthesis after 90-second flushing with $N_2$ in the light.

* Values are in thousands of counts/min/cc wet-packed cells, determined as total plate counts of the original algal suspension in ethanol.

TABLE 2

STABLE $CO_2$ FIXATION AND ACTIVITY OF THE ACETONE-SOLUBLE FRACTION*

| Killing Procedure | Stable Fix. in Total Original Suspension | Activity of Acetone-soluble Fraction† | Relative Amount of Phosphorous Esters in Acetone-soluble Fraction (Per Cent) |
|---|---|---|---|
| Acetone (−36° C.) | 3,099 | 650 | 44.6 |
| $NH_2OH$ followed by acetone (−35° C.) | 5,517 | 4,387 | 88.6 |
| Acetone (−35° C.) followed by $NH_2OH$ | 3,490 | 3,320 | 83.5 |

5-second photosynthesis after 90-second flushing with $N_2$ in the light.

* Values in thousands of counts/min/cc wet-packed cell.
† Practically all the radioactivity which does not appear in the acetone is extracted by $H_2O$ immediately following the acetone extraction.

The discovery of the hydroxylamine effect encouraged us to develop a method for the study of the initially unstable complex, or its more stable derivative(s), by paper chromatography. Preliminary experiments were performed to see whether the labile complex survives contact with the solvent systems that we were using. Aliquots of two *Scenedesmus* suspensions killed by subzero acetone both with and without previous addition of hydroxylamine were pipetted onto a sheet of chromatography paper. One spot of each series was cut out and burned in a glass bomb. The remaining spots were wet either with phenol or with the butanol–propionic acid mixture used in the normal chromatographic separation. After drying the paper in a stream of cold air (total contact time with solvent 5–10 minutes), these spots were cut out and burned in a similar way. While the aliquots taken of extracts with alcohol or acetone upon contact with phenol suffered a loss of about 30 per cent of the activity compared with the spot which had had no solvent contact, nothing was lost from those extracts involving the hydroxylamine. The butanol–propionic acid mixture, on the other hand, leads to practically the same loss (about 10 per cent) from both types of extracts. This suggested one-dimensional chromatography, using only phenol. To improve the separation, the paper was cut as described elsewhere.[4]

Two series of experiments were performed using *Scenedesmus* cells which had been photosynthesizing in $C^{14}O_2$ for 5 seconds. In one, the cells were killed and extracted with alcohol (Fig. 1) and in the other with acetone (Fig. 2). In each series the organic insoluble residue was extracted with water. The two extracts (organic solvent and water extracts) were chromatographed separately in one

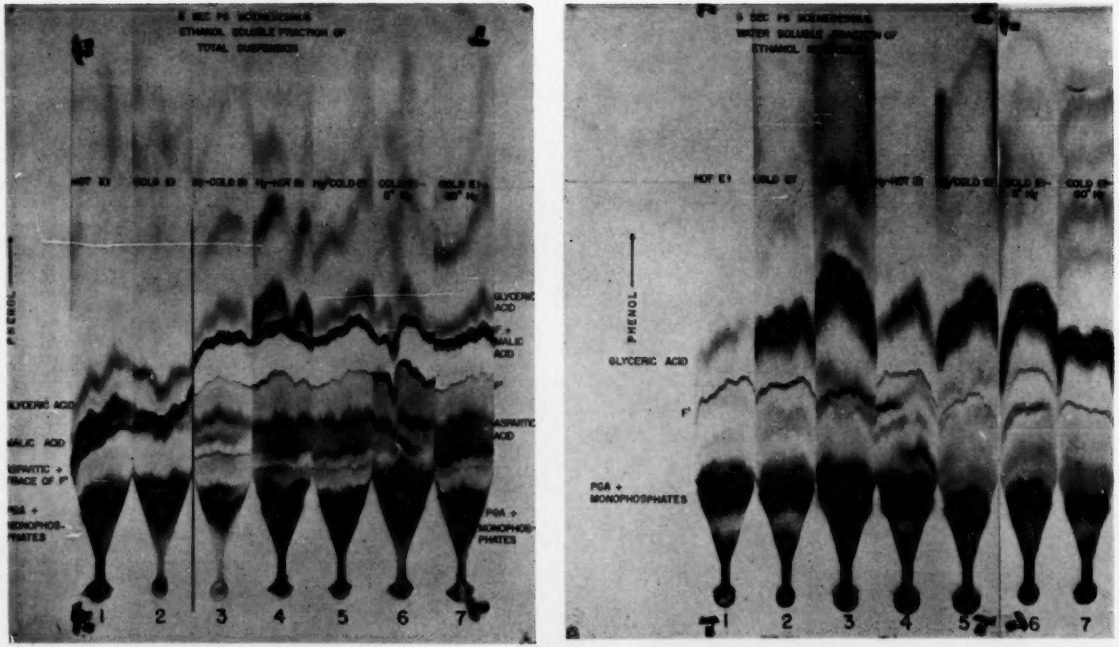


FIG. 1. Radioautograph of one-dimensional phenol chromatograms of various alcohol and water extracts of *Scenedesmus* cells which have been photosynthesizing in $C^{14}O_2$ for 5 seconds and then killed in a variety of ways. *Left*, the ethanol extracts; *right*, the water extracts of the alcohol-insoluble residue. *1*, hot alcohol; *2*, cold alcohol ( −35° C.); *3*, hydroxylamine added to cell suspension to make it 0.01 *M* in hydroxylamine and then immediately killed in cold alcohol ( −35° C.); *4*, hydroxylamine added to cell suspension to make it 0.01 *M* in hydroxylamine and then immediately killed in hot alcohol; *5*, algae killed by dropping into a 0.01 *M* solution of hydroxylamine in cold ethanol ( −35° C.); *6*, cold alcohol ( −35° C.) followed in 5 seconds by the addition of hydroxylamine; *7*, cold alcohol ( −35° C.) followed in 60 seconds by the addition of hydroxylamine.

dimension, using phenol. All the chromatograms of material to which hydroxylamine was added either before, during, or after the killing process, showed two fluorescing spots which coincided with two blackened bands on the exposed X-ray films. The faster-moving spot ($F$) runs just behind malic acid and is not, in general, well separated from it, even after an 18-hour run. The other fluorescing compound ($F'$) lies between malic and aspartic acid. In the control experiments (without hydroxylamine) with cold and hot ethanol as well as with cold acetone

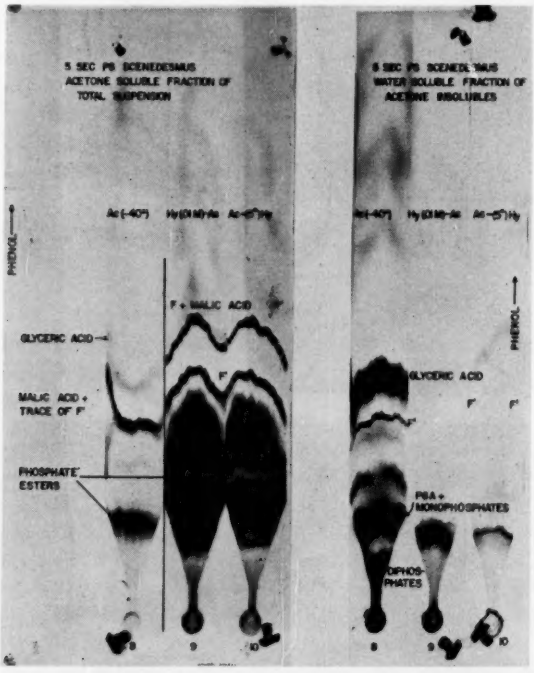


FIG. 2.—Radioautograph of a one-dimensional phenol chromatogram of various acetone and water extracts of *Scenedesmus* cells which have been photosynthesizing in $C^{14}O_2$ for 5 seconds and then killed in a variety of ways. *Left*, the acetone extracts; *right*, the water extracts of the acetone-insoluble residue. (*8*), killed by cold acetone (−40° C.); *9*, hydroxylamine added to cell suspension to make it 0.01 *M* in hydroxylamine and then immediately killed in cold acetone (−40° C.); *10*, killed in cold acetone (−40° C.) followed in 5 seconds by the addition of hydroxylamine.

there is only a trace of a lower band which might correspond to $F'$. From phenol chromatograms of the alcohol-soluble components (Fig. 1, *left*) it was possible to estimate the amount of $F$ and $F'$ (in Experiment 6) at about 9 per cent of the total extracted into alcohol. Since this extract contains about half the total $C^{14}$ fixed, the amount of $F$ and $F'$ which survives the chromatography constitutes about 5 per cent of the total.

Two-dimensional chromatograms do not seem to show the upper band ($F$) (Fig.

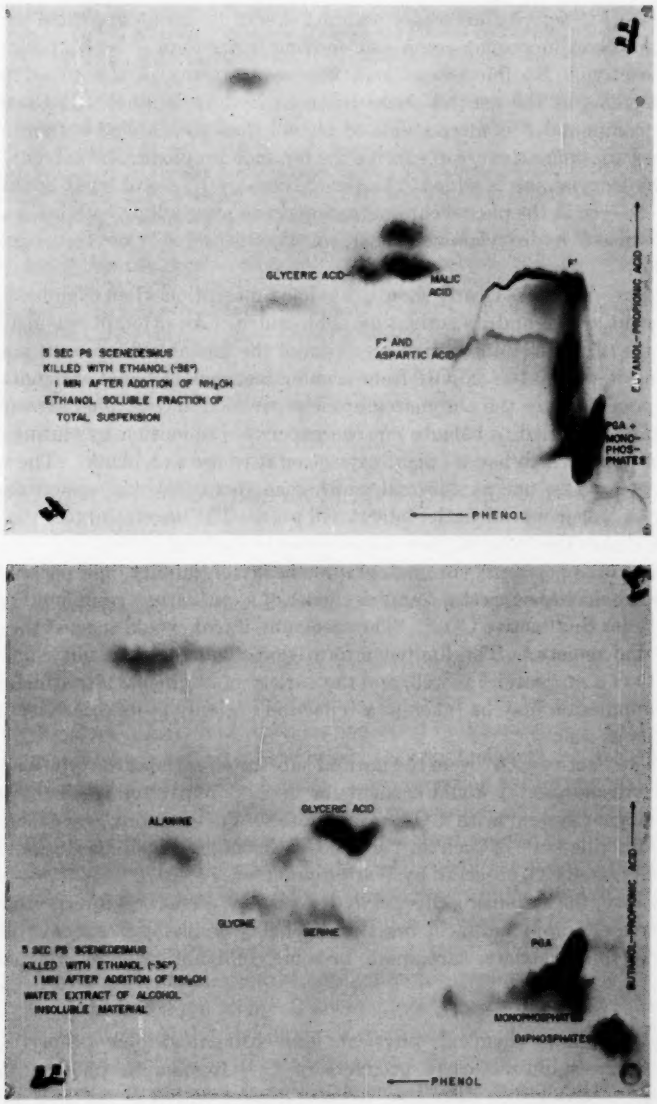


FIG. 3.—Radioautographs of chromatograms of 5-second photosynthesis experiments using *Scenedesmus;* light turned off and hydroxylamine added; after 1 minute ethanol (−36° C.) added. *Top,* ethanol extract; *bottom,* water extract of alcohol-insoluble material.

3); it appears to have been destroyed by the butanol–propionic acid system. In the hydroxylamine experiments, however, some of the lower spot (*F'*) remains. It often separates into two bands (*F'* and *F"*, with the latter running close to aspartic

acid). Its ($F'$) activity decreases within 2 weeks to about one-third its original value. Another fluorescing compound moving faster than $F$ is also present but is not radioactive. No fluorescent area was seen on any of the two-dimensional chromatograms of the control series (without hydroxylamine). It thus appears that the compound $F$ is more stable to phenol than to acidified butanol; whereas $F'$, at least to some extent, can survive the butanol–propionic acid solvent, particularly if hydroxylamine is added. However, because traces of what appears to be $F'$ are to be seen in the phenol chromatograms even after killing with boiling ethanol in the absence of hydroxylamine (Fig. 1, col. *1*), it is probably not the original labile material.

The fluorescing areas clearly show ultraviolet absorption when examined between a fluorescent screen and its activating light source. An attempt was made to determine the ultraviolet absorption spectrum of the unstable fluorescent material $F$, by eluting it with 0.1 $N$ NaOH from a one-dimensional phenol chromatogram as soon as possible after the chromatographic separation and development of the exposed film. We tried to balance out the paper contamination by eluting an equal weight of paper which had no plant extract on it to use as a blank. The spectrum showed two peaks, one at 255 and another at about 285 m$\mu$, sometimes with a shoulder at longer wave lengths (about 340 m$\mu$). The uncertainty of the balance, however, makes the value of these measurements doubtful as yet.

The time of appearance, chromatographic behavior, lability, and particularly the fluorescent behavior suggest a complex carrier of a one-carbon compound as a likely possibility for this "active $CO_2$." The spectrum, if real, would suggest the presence of a pterino moiety.[5] The limited information available does not eliminate the possibility of a nucleotide[6] as well, and the variety of fragments into which it breaks upon manipulation may be taken as a reflection not only of its reactivity but of its complexity as well.

If such an "active $CO_2$" were the natural substrate for the carboxylation reaction[7] of carboxydismutase,[8] it would account for the apparently too small turnover rate of this enzyme system with $CO_2$ as substrate.[9] Furthermore, a particular sensitivity to fluoride ion[10] of such an "active $CO_2$" complex could also account for the fluoride-liberated $CO_2$ observed by Warburg *et al.*[11]

In any case, the stabilizing effect of hydroxylamine seems best interpreted in terms of an extremely rapid and facile reaction, probably involving a carbonic-phosphoric anhydride, thiocarbonate, carbamate, or some combination or reactive derivative of these.

*Summary.*—Some chemical, physical, and chromatographic properties of the hydroxylamine-stabilized early products of $CO_2$ fixation in photosynthesis are described. Although no definitive structural information is yet available, these properties, together with the biochemical context in which the material appears, make possible some likely suggestions about the nature of the substances.

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‡ Guest research worker, 1956–57.

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## *THE NATURE OF BOND ORBITALS AND THE ORIGIN OF POTENTIAL BARRIERS TO INTERNAL ROTATION IN MOLECULES*

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Twenty years ago Kemp and Pitzer[1] discovered that the relative rotation of the two methyl groups about the carbon-carbon bond in ethane is not completely free but is restricted by a potential barrier about 3.0 kcal/mole high, with three maxima and three minima in a complete rotation, corresponding to the trigonal symmetry of the methyl groups. It was soon found that rotation about single bonds is restricted in many molecules, and many experimental values for the height of the potential barrier have been obtained by the analysis of thermodynamic quantities (entropy, heat capacity) and especially by the methods of microwave spectroscopy.

Progress has been slow in the development of a satisfactory theory for the potential barriers, despite the efforts of several investigators. The theoretical attack was begun by Eyring,[2] who made approximate quantum-mechanical calculations of the interactions of the hydrogen atoms of the two methyl groups. The various suggestions and calculations about the importance of van der Waals repulsion between attached groups, electrostatic interactions of the charge distributions in the bonds between the two carbon atoms and the attached groups, and intrinsic lack of cylindrical symmetry in the axial chemical bond itself have been summarized recently by Wilson,[3] who tested these hypotheses by comparison with the values of the potential barriers that have been determined experimentally and reached the conclusion that the potential barriers to internal rotation "must in some way be an inherent property of the axial bond itself and not due in any substantial measure to direct forces between the attached atoms or those parts of the electron distribution which are out on the attached bond any considerable distance." References to the

earlier theoretical papers and to the sources of most of the values of potential barriers mentioned later in this paper are given by Wilson.

I have developed a simple theory of these potential barriers, described in the following paragraphs. According to this theory, the potential barriers are not a property of the axial bond itself, but result from the exchange interactions of electrons involved in the other bonds (adjacent bonds) formed by each of the two atoms, as determined by the overlap between the parts of the adjacent bond orbitals that extend from each of the two atoms toward the other.

*Concentration of Bond Orbitals.*—The four bonds formed by a carbon atom can be discussed with use of tetrahedral orbitals formed by hybridization of the *s* orbital and the three *p* orbitals of the neon shell.[4, 5] The description of these orbitals as $sp^3$ tetrahedral orbitals is, however, only a first approximation. Even for the hydrogen atom, for which the 1*s* orbital is far more stable than any other orbital, hybridization occurs on bond formation. This effect has been described[5] as *concentration* of the bond orbitals. For example, in the approximate variation treatment of the hydrogen molecule by Wang[6] and the corresponding treatment of the hydrogen molecule-ion by Finkelstein and Horowitz,[7] use is made of an orbital that has the form of a 1*s* orbital but with changed effective nuclear charge, found by minimization of the energy to be 1.17 and 1.23, respectively. The change in the value of the effective nuclear charge is equivalent to hybridizing the 1*s* orbital of the hydrogen atom with the 2*s*, 3*s*, and higher orbitals, including *s* orbitals in the continuum. These higher *s* orbitals make a total contribution of 2 per cent to the hybrid bond orbitals. Also, a significant improvement in the approximate wave function can be obtained by adding to the *s* orbital a term containing a cosine function, which we may describe as a *p* orbital. This *p* term is a hybrid of 2*p*, 3*p*, and higher *p* orbitals. The effective nuclear charge that minimizes the energy is about twice that for the *s* orbital, leading to approximately the same spatial extent. It was shown by Rosen[8] for the hydrogen molecule and by Dickinson[9] for the hydrogen molecule-ion that the energy is minimized when the *p* terms occur in the wave function with coefficients 0.10 and 0.15, respectively, corresponding to 1 and 2 per cent of *p* character in the hybrid orbitals. These amounts of *p* hybridization are found, despite the fact that the promotion energy for the *p* terms is about 330 kcal/mole, slightly larger than the ionization energy of the hydrogen atom, 313 kcal/mole. The promotion energy for the higher *s* terms is also about 330 kcal/mole. (I am indebted to Dr. Brian Gray for carrying out the expansions of the wave functions in the set of hydrogen-atom functions with effective nuclear charge unity, and evaluating the promotion energies.)

We may accordingly expect that the bond orbitals of the carbon atom will be found on careful examination to be hybrids with some *d*, *f*, etc., character, in addition to the main $sp^3$ tetrahedral character. A rough estimate of the contributions of *d* and *f* terms to the hybrid bond orbital can be made by assuming the bond energy to be proportional to the strength of the bond orbital, as determined by the angular distribution,[5] and taking the promotion energy for both *d* and *f* as equal to the ionization energy of the carbon atoms. This calculation leads to coefficients approximately 0.15 for both the *d* term and the *f* term, corresponding to about 2 per cent *d* character and 2 per cent *f* character in the tetrahedral orbitals.

The calculation is made in the following way. The bond orbital is taken to be

$$\Psi = \alpha s + \beta p_z + \gamma d_z + \delta f_z, \tag{1}$$

in which $s$, $p_z$, $d_z$, and $f_z$ are the orbitals with maximum values along the $z$-axis (the bond direction); normalized to $4\pi$, their angular parts are

$$s = 1,$$

$$p_z = \sqrt{3}\cos\theta,$$

$$d_z = \sqrt{5/4}\,(3\cos^2\theta - 1),$$

$$f_z = \sqrt{7/4}\,(5\cos^3\theta - 3\cos\theta)$$

The value of this function in the bond direction, called the "strength of the bond orbital," is

$$S = \alpha + \sqrt{3}\beta + \sqrt{5}\gamma + \sqrt{7}\delta \tag{2}$$

The effective bond energy is assumed to be the product of $S$ and a bond-energy parameter $b$, corrected by subtracting the terms $\gamma^2 P_d$ and $\delta^2 P_f$, in which $P_d$ and $P_f$ are the promotion energies for the $d$ and $f$ terms:

$$\text{Bond energy} = bS - \gamma^2 P_d - \delta^2 P_f \tag{3}$$

The value of $b$ is taken to be 36 kcal/mole, which leads to bond energy 82 kcal/mole, corresponding to an average bond formed by a carbon atom (C—C, 82.3; C—H, 99.6; C—O, 81.0; etc.). The promotion energies are taken to be $P_d = 300$ kcal/mole and $P_f = 350$ kcal/mole, somewhat larger than the ionization energy of carbon, 260 kcal/mole, as indicated by consideration of the energies of the lowest $s$, $p$, $d$, $f$ Epstein functions for the hydrogen atom.[10] In accordance with the minimum-energy theorem of quantum mechanics, we vary the parameters $\gamma$ and $\delta$ to make the bond energy a maximum. (A satisfactory approximation is to take $\alpha = {}^1/_4$; then $\beta^2 = {}^3/_4 - \gamma^2 - \delta^2$.) It is found that both $\gamma$ and $\delta$ then have the value 0.15, with $\beta = 0.84$, and the bond orbital has the simple angular dependence

$$\psi = 0.33 + 0.85\cos\theta + 0.50\cos^2\theta + \cos^3\theta \tag{4}$$

This function has a maximum value (bond strength) of 2.68, which is 34 per cent greater than for an $sp^3$ orbital—a large effect of 2.25 per cent $d$ character and 2.25 per cent $f$ character. The nodal cone lies at 113.8° from the bond direction, only slightly shifted from the $sp^3$ value 109.5°. The negative lobe contains 7 per cent of the distribution function $\psi^2$, considerably less than the 11 per cent for $sp^3$ orbitals. The value of the function is larger than that of the $sp^3$ tetrahedral orbital from the bond axis out to 44°, and then becomes smaller, being about two-thirds as great (less than half the electron density) from 65° to 140°. The concentration of the orbital in the bond direction is well described by the average value of $\cos\theta$ (weighted with the distribution function $\psi^2$), which is 0.771 for function 4, and only 0.312 for an $sp^3$ orbital.

The hybrid orbital has cylindrical symmetry, and accordingly the introduction of $d$ character and $f$ character in the axial bond itself does not lead to an interaction dependent on the relative azimuthal orientation of the two groups.

*The Interaction of the Concentrated Hybrid Orbitals.*—The interaction of the two

electrons occupying one adjacent bond orbital of each of two atoms forming an axial bond can be discussed by expanding the adjacent bond orbitals in terms of orbitals described with reference to the axial bond direction, which may be taken as the $z$-axis. The interaction energy of these two electrons (which with two other electrons, on adjacent atoms, form bonding electron pairs) includes the resonance integral[11] with the coefficient $-1/2$ (rather than the $+1$ that occurs in the energy for a bond), and accordingly the negative sign of the resonance integral itself leads to repulsion between the two electrons and maximum stability when the overlapping is a minimum. In the expansion in functions about the bond direction there will occur $\sigma$ terms (independent of the azimuthal angle $\varphi$), $\pi$ terms (with factor $\cos\varphi$ or $\sin\varphi$), $\delta$ terms ($\cos 2\varphi$), and $\phi$ terms ($\cos 3\varphi$). The interaction of two functions with $\sigma$ and $\phi$ character gives rise to an interaction energy proportional to $\cos 3\varphi$, where $\varphi$ represents the relative azimuthal orientation of the two groups. This is the term of lowest order that occurs if one of the groups has trigonal symmetry (such as the methyl group).

A very rough estimate of the height of the potential barrier may be made. The $f$ term in an adjacent bond orbital as given in equations (1) and (4), which has its axis in a direction 70.5° from the $z$-axis determined by the axial bond, can be expanded in terms of $f$ orbitals referred to this $z$-axis, and it is found that the coefficient of the normalized $f$ orbital $\sqrt{35/8}\sin^3\theta\cos 3\varphi$ is $0.664\delta$, or 0.100 for $\delta = 0.150$. The strength of this orbital is 2.09, and hence it contributes 0.209 to the strength of the hybrid bond orbital. We may expect the corresponding term in the resonance integral to be approximately $(0.209/1.732)^2$ times as great as for two $\pi$ orbitals, as in the carbon-carbon double bond, which is about 61 kcal/mole.[12] Taking into account also the factor $-1/2$ for non-bonding rather than bonding interaction, we obtain 0.44 kcal/mole. Each of the three C—H bonds of one methyl group interacts with each of the three of the other group; hence we predict that the potential barrier for ethane and substituted ethanes (forming single bonds only) should be about $9 \times 0.44 = 4.0$ kcal/mole high. This value is completely unreliable, and no significance should be attached to its rough agreement with the experimental values 2.7–3.0 kcal/mole for ethane, 3.30 for $CH_3CH_2F$, 3.18 for $CH_3CHF_2$, and 3.3 for propane.

The smaller value 2.56 kcal/mole found for $CH_3CHOCH_2$ shows the decrease that we would expect to result from the change in bond angles caused by the three-membered ring. If the change is distributed equally over the five other bond angles, increasing them from 109.5° to 120°, the expansion of the $f$ orbital introduces the coefficient $0.514\delta$ in place of $0.664\delta$ and hence leads to a predicted barrier 77 per cent as great as for unstrained substituted ethanes, in good agreement with experiment ($2.56 = 0.77 \times 3.32$).

There are other predictions that can be made on the basis of this theory, and there is further good agreement with experiment. First, it is predicted that the staggered configuration of substituted ethanes and similar molecules is the stable one. The staggered configuration has been verified for $CH_3CH_2Cl$, $CH_3CF_3$, $CH_3SiH_3$, $CH_3SiH_2F$, $CH_3SiF_3$, and several other molecules.

Also, the height of the barrier changes from molecule to molecule in the predicted way. The barrier interaction involves the same integral over the radial parts of the wave functions as the axial bond itself, and it would accordingly be expected that

for molecules in which the bond orbitals have similar hybrid character the barrier height would be a constant fraction of the bond energy. In particular, for different substituted ethanes essentially the same barrier would be found, provided that the substituent groups are not large enough to cause steric effects, which would increase the barrier height. Approximate constancy is observed for ethane and substituted ethanes, as mentioned above.

The energy of a carbon-silicon bond and that of a carbon-germanium bond are about three-quarters as great as that of a carbon-carbon bond, and it would accordingly be predicted that the height of the barrier in molecules containing these bonds would be about three-quarters as great, that is, about 2.3 kcal/mole; observed values are 1.70 for $CH_3SiH_3$, 1.56 for $CH_3SiH_2F$, 1.32 for $CH_3SiHF_2$, and 2.5 for $CH_3GeH_3$ (perhaps uncertain). (Professor E. B. Wilson, Jr., has just written me that the value for methyl germane is probably 1.2.)

Only bond orbitals, and not orbitals for unshared pairs, hybridize with *f* character, and accordingly we predict that an OH group would interact with a methyl group one-third as strongly as a methyl group itself would, and an $NH_2$ group would interact two-thirds as strongly; for $CH_3OH$ and $CH_3NH_2$ we predict barrier heights about 1.0 and 2.0 kcal/mole, respectively; the observed values are 1.07 and 1.90.

We may describe the double bond as involving two tetrahedral orbitals of each of the two bonded atoms; that is, as two bent single bonds.[5] It would then be expected that the interaction energy of a methyl group with a carbon atom forming a single bond and a double bond would be somewhat smaller than that between two methyl groups, that is, somewhat smaller than 3 kcal/mole. The observed barrier height for methyl ethylene ($CH_3$—CH=$CH_2$) is 1.98 kcal/mole, in agreement with expectation. Similar values are found in the related substances $CH_3$—CH=C=$CH_2$ (1.59) and $CH_3$—CH=CHF (2.15). Configurations have not been determined for these substances; we may predict that a hydrogen atom of the methyl group will be staggered with respect to the single bond on the other carbon atom.

In aldehydes and related substances the height of the potential hump is somewhat less than in methyl ethylene: 1.15 kcal/mole for $CH_3CHO$, 1.08 for $CH_3CFO$, 1.35 for $CH_3CClO$, and 1.27 for $CH_3COCN$. Configurations in agreement with our prediction have been reported for the first three, a methyl hydrogen atom being staggered with respect to the single bond formed by the other carbon atom.

For acetic acid, $CH_3COOH$, in which the bond from carbon to the OH group has about 35 per cent double-bond character and that to the oxygen atom about 65 per cent double-bond character, as determined from the interatomic distances, we would predict for the potential hump a value about one-third that in acetaldehyde. This prediction is borne out by experiment, the height of the barrier being 0.48 kcal/mole.

For nitromethane, $CH_3NO_2$, and methyl difluoroborane, $CH_3BF_2$, symmetry requires that the potential barrier correspond to a sixfold axis. The experimental values for the barrier height are very small, 0.006 and 0.014 kcal/mole, respectively. These very small values show that the contributions of the corresponding orbitals to the hybrid bond orbitals are also very small. The orbitals that could give rise to a potential barrier proportional to $\cos 6\varphi$ are the *i* orbitals, with azimuthal quantum number $l = 6$. Because of the many nodal cones and the large promotion energy

of the $i$ orbitals, it is to be expected that they would not make any important contribution to the hybrid bond orbitals.

The general qualitative agreement with experiment provides support for the theory that the potential barriers to internal rotation result from the interaction of adjacent hybrid bond orbitals with a small amount of $f$ character. The magnitude of the potential barriers, about 4 per cent of the energy of the axial bond in case that there are three interacting bonds on each of the two atoms and proportionately less for a smaller number of bonds, is also reasonable. A detailed quantum-mechanical treatment of restricted rotation carried out along the lines sketched here should yield results that would permit a detailed test of the theory to be made; in the meantime I believe that the above simple treatment and the extensive empirical support of the theory provide justification for it.

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## *IDENTIFICATION OF BLOOD CHARACTERISTICS COMMON TO ALCOHOLIC MALES**

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In 1947 our laboratory began the intensive study of alcoholism, on the presumption that individual differences in metabolism are basic to the etiology of the disease and that "no psychological stresses can make an individual an alcoholic unless he has inherited a metabolic pattern which renders him susceptible."[1] This working hypothesis—so designated at the time—has held our constant interest.

The difficulties of testing this hypothesis have been great, mainly because at the start almost complete ignorance existed as to how one individual might differ from another metabolically. Very few investigators have had any specific interest in gathering this type of information. Recently the senior author has attempted to assemble in a book entitled *Biochemical Individuality*[2] the available material on the

subject, most of it derived, however, from the work of those who had no interest in individuality as such.

At the time we embarked upon the study of alcoholism we had in mind the possibility of finding metabolic earmarks that might characterize alcoholics, and we indicated: "If the potential addict could be forewarned, avoidance of alcoholism would be made relatively easy."[1] This statement was made before we had developed the genetotrophic concept,[3] which emphasizes the potentialities of completely adequate nutrition for the prevention of the disease.[4]

The present discussion has to do with the progress we have made in finding metabolic earmarks which might be useful in detecting alcoholism-prone individuals before they become addicts.

Recently we have completed an experimental study of 53 male alcoholics, twenty-seven to sixty-two years of age, and 41 male control individuals, twenty-one to forty-eight years of age, who, although they consume some liquor, have avoided exhibiting alcoholic tendencies. The alcoholic subjects were studied in co-operation with the Austin State Hospital. Two preliminary studies[5, 6] had been very helpful in directing attention to items which gave promise of being significant in this connection. Two samples of venous blood were drawn on separate days under basal conditions from each of the 94 individuals. Cell counts and analyses were made on each sample. The results from the two samples were averaged and used in the computations.

The items listed in Table 1 were found to be significantly different for the controls and the alcoholics. Serum magnesium was higher in the alcoholic group, but the *P*-value was only 0.10. Figures 1 and 2 show the distribution of the values for two items in the list: serum potassium, which showed the most consistent differences, and the blood sugar levels, which showed the least consistent differences among the seven items.

TABLE 1

BLOOD VALUES FOUND TO BE DIFFERENT FOR CONTROLS AND ALCOHOLICS

| | "Normal" Range (Albritton) | Control Average | Alcoholic Average | *P*-Value |
|---|---|---|---|---|
| Total leukocytes | 3,480–14,840/mm³ | 6,450 | 8,420 | 0.001 |
| Lymphocytes | 1,000–4,800/mm³ | 1,520 | 2,020 | 0.001 |
| Eosinophils | 50–700/mm³ | 156 | 355 | 0.001 |
| Blood sugar | 76–96 mg/100 ml | 96 | 106 | 0.01 |
| Serum sodium | 132–144 meq/1000 ml | 134 | 141 | 0.001 |
| Serum potassium | 3.6–4.8 meq/1000 ml | 3.8 | 4.95 | 0.001 |
| Serum calcium | 4.0–5.5 meq/1000 ml | 4.67 | 5.18 | 0.001 |

Urine samples were collected from the same 94 individuals at four regular times on each of two separate days. Equal volumes of these specimens were pooled, and the two composite samples from each individual were analyzed for a series of items. The results from the two pooled samples were averaged and used in the computations. It was not feasible under our conditions of operation to obtain 24-hour urine samples from these large groups.

A notable difference between the alcoholics and the controls was the level of creatinine in the urine on a volume basis. The alcoholic average was a little less than half that of the controls, and there was only one alcoholic in the whole group who exhibited a creatinine level higher than the mean of the control group. A

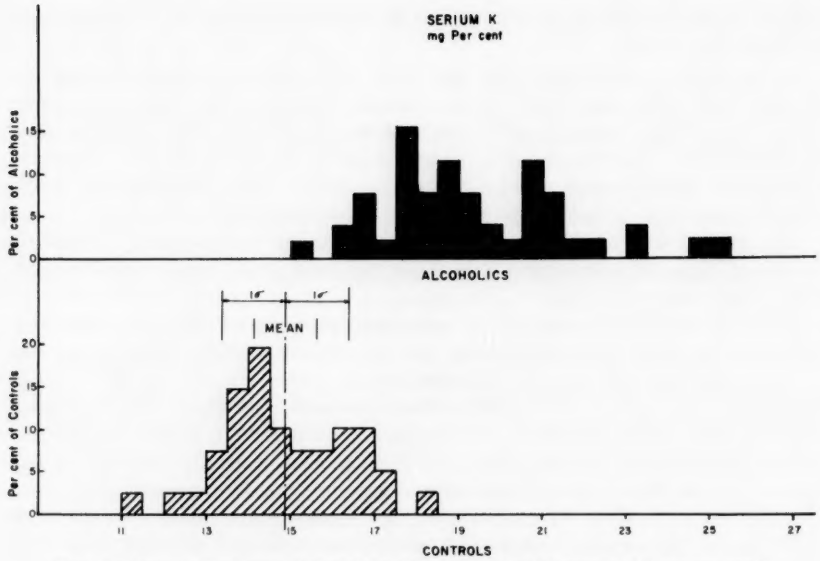


FIG. 1.—Serum potassium. Percentage distribution (to the nearest 0.5 mg per cent)

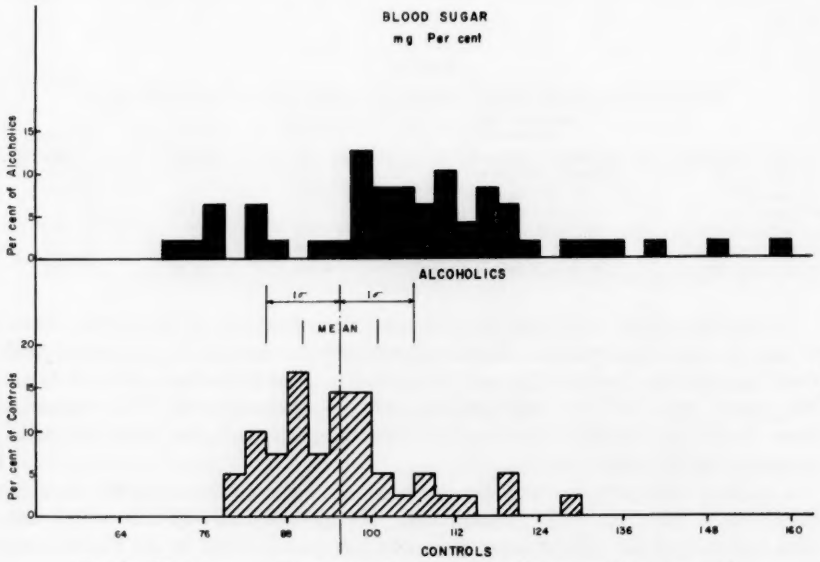


FIG. 2.—Blood sugar. Percentage distribution (to the nearest 3 mg per cent)

separate study in which three consecutive 24-hour samples from a smaller group of alcoholics were analyzed indicated that their daily creatinine excretion is about normal and that the lower creatinine level in alcoholics is due primarily to their urine being more dilute.

The urinary items which appeared significantly different for the two groups are given in Table 2. Urinary pH was measured but not found to be significantly different for the two groups.

TABLE 2

SIGNIFICANT URINARY ITEMS: CONTROL VERSUS ALCOHOLICS

| | Controls | Alcoholics | *P*-Values |
|---|---|---|---|
| Creatinine mg/ml (largely a measure of urine concentration) | 2.03 | 0.97 | 0.001 |
| Hippuric acid, chromatographic spot area/mg creatinine | 0.20 | 0.27 | 0.01 |
| Sodium mg/mg creatinine | 2.26 | 3.25 | 0.001 |
| Potassium mg/mg creatinine | 1.09 | 1.99 | 0.001 |
| Chloride mg/mg creatinine | 3.89 | 5.94 | 0.001 |

Because urinary variations are larger and subject to more environmental influences, we place somewhat greater confidence in the blood data. The urine data appear meaningful, however, as shown by the statistical analysis and the fact that the urine data and the blood data are in agreement with respect to the status of about 95 per cent of the individuals, when they are judged on the basis of their "alcoholism indexes," described later.

With these data at hand, it seemed possible to devise a test which could be used to classify *individuals* as to their likeness or unlikeness to the alcoholic group as a whole. To do this, we have assigned to each of the values from each individual an "index number" which indicates how many half-standard deviations the value is on the "alcoholic side" of the control mean. In the case of every item except the urinary creatinine level, the high side is the "alcoholic side"; in this case the values from the alcoholics fall on the low side. Negative index numbers are assigned to values when they are on the opposite side of the control mean from the position of the alcoholic mean.

In order to compute the "alcoholism index" of each individual, we averaged the indexes for each of the twelve individual items listed in Tables 1 and 2. By this means we obtained composite indexes of from $-1.8$ up to $+2.4$ (average $+0.07$) for the control group, and from $+0.7$ up to $+8.3$ (average $+3.67$) for the alcoholic group. Only four controls had composite indexes of $+1.5$ or higher, and only two alcoholics had indexes lower than $+1.8$, so there was relatively little overlapping. If we were able to measure alcoholism-proneness successfully, it would not be surprising if we found among "non-alcoholic" individuals some who were innately more susceptible than some alcoholics whose training and environment had been conducive to the development of the disease.

To test the validity of the "alcoholism indexes," we have computed, without any adjustments, the composite indexes for 8 women (4 alcoholics and 4 controls), for whom we had collected data. None of these data, however, were used in establishing norms. On this basis the indexes of the four alcoholic women were 2.8, 1.8, 3.8, and 3.9, respectively, while the indexes of the control women were 0.1, 1.2, $-0.6$,

and 1.2. When this test based on data obtained from males was applied without change to females, we anticipated that the results might be less consistent than they were. It is apparent that alcoholic males and females have characteristics in common and that in these 8 female cases the test appears to be applicable.

To test further the validity of the "alcoholism indexes," we compared these individual composite indexes with independent "severity" ratings given groups of the same individuals, *40* and *29*, respectively, by an attending physician and a psychiatric nurse. These psychiatric ratings were from 1 for mild cases up to 5 for the most severe. The results showed that our alcoholism indexes and each of the psychiatric ratings correlated significantly; the *P*-value in each case was 0.05.

These facts point with a high degree of certainty to the conclusion that the items which we have measured are directly related to the disease alcoholism. A serious question arises as to whether the observed differences have to do with alcoholism-proneness or whether excessive alcohol consumption over a period of years has induced the same changes in the victims. While we are not in a position to answer this question with certainty, we can, on the basis of what we already know about individual patterns and their stability, indicate with confidence that alcohol consumption is not wholly responsible for the differences observed. Probably this factor enters appreciably into some of the measurements, but that it is minor is indicated by a large amount of general and specific evidence. The general evidence involves considerations of anatomy, composition, endocrine patterns, enzyme levels, excretion patterns, pharmacological responses, variable nutritional needs, etc., which have been summarized in the book *Biochemical Individuality*.[2]

Specific evidence that the hematological components of the test are genetically determined was obtained by Rosahn and Casey,[7] who studied five healthy men over a period of a year and six healthy rabbits over a period of two years and found that in each species there was statistically significant differences between the individuals with respect to red cell counts, total leukocytes, neutrophils, basophils, eosinophils, lymphocytes, and monocytes. They suggested that "each individual has a characteristic and typical blood formula which is largely determined by genetic factors." It is interesting that 80 per cent of the known alcoholics in our group could be correctly classified on the basis of our test, using only the three blood cell values which we determined.

In a doctoral dissertation which has not been published in detail, R. W. Shideler[8, 9] has found evidence by repeated analyses of samples from the same nine normal young men, that each individual exhibits a distinctive pattern with respect to the levels of sodium, potassium, calcium, magnesium, and phosphorus in saliva, plasma, red blood cells, and urine. With respect to most of these twenty items, there is clear evidence that certain individuals stand out as distinctly different from others. Here again the only adequate explanation is that genetic differences are responsible. Some may be inclined to dismiss urinary differences in mineral components by remarking that if one individual has more salt in his urine than another, it simply means that he has consumed more salt. There is nothing wrong with this statement except the word "simply." There must be physiological reasons, based, for example, on tremendous differences in adrenal cortices, why some "normal" people consume much more salt than others.

That the concentrations of various organic substances in urine vary widely from

individual to individual, even among babies, has been demonstrated in our laboratory[10, 11] over a period of years. In experimental animals the differences may be enormous, up to tenfold or more, when the animals are consuming exactly the same food. Closely inbred strains show many strong resemblances not shown by animals of heterogeneous ancestry, and it is evident that genetic factors are highly important. In human beings we know that some distinctive features of excretion pattern remain unchanged over a period of years—in one case that happens to have come under observation this was true even after a pregnancy and parturition. On the basis of these facts we regard the distinctive urinary levels as having a substantial genetic basis.

Underlying all these data is the fundamental fact, which is becoming clearer with the advance of biochemical genetics, namely, that gene differences (major or minor) give rise to enzyme differences in specific tissues, and these in turn must be largely, if not wholly, responsible for the basic differences in our individual body chemistries.

We do not take the position that our norms can be universally applied or that we have at hand a complete and dependable test for alcoholism-proneness which calls for no further investigation. Actually, there are several additional items not covered in this study which are at least promising: salivary sodium,[5] urinary uric acid,[5, 6] serum copper,[12] and serum amylase.[13] Uric acid requires further study with a completely reliable method. The elevated serum copper level (probably like a number of other individual items) is not specific for alcoholism. In this case elevated values are also found in pregnancy, schizophrenia, and a variety of infections.

We are of the opinion that the test for alcoholism-proneness as we have developed it can be applied immediately at the research level, and from such investigations a highly dependable test will develop. At the present time we regard the items we have measured as convenient earmarks rather than basic differences which necessarily have directly to do with alcoholism itself. In other words, we would consider it naïve to expect that lowering the respective levels in the blood and urine, even if it were possible, would constitute a cure of the disease. We suspect that when the crucial differences between alcoholism-prone and other individuals are found, these differences may involve large metabolic differences between specific structures which function in the regulation of appetites. What we have observed may well be minor metabolic reflections of these larger specific differences.

In conclusion we can say that, on the basis of accumulating evidence, we are of the opinion that alcoholism can probably be prevented even in severely alcoholism-prone individuals if they can be identified and if adequate account is taken of their nutritional needs, which may, for specific items, be far from the values commonly tabulated as satisfying the (hypothetical) standard or normal man.[14] Nutritional help may involve vitamin supplementation, but, in addition, other items such as minerals and amino acids (including glutamine[15]) must be considered.

We wish to acknowledge with gratitude the invaluable co-operation of the 57 alcoholics and the 45 controls, as well as the help of the superintendent and staff of the Austin State Hospital, and the following individuals who helped with analytical work: Ruth Kellogg, Douglas Kellogg, Edith Wilson, Ewa Abeman, and Ivan Roth. We are also grateful to Professors Wayne Holtzman, University of Texas, John Tukey, of Princeton, and Dr. Jerome Cornfield, of the U.S. Public Health Service,

who gave statistical advice but who should not be held responsible for the exact procedures which we utilized.

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## *AN EXAMPLE OF A NONNORMAL DISTRIBUTION WHERE THE QUOTIENT FOLLOWS THE CAUCHY LAW*

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Let $x$ and $y$ be two independently and normally distributed variates with zero means and the common variance; it is then well known that the quotient $x/y$ follows the Cauchy distribution symmetrical about zero. It has been conjectured that this is a unique property of the normal distribution and that it is possible to obtain a characterization of the distribution by this property. In the present note we show that this conjecture is not true, and we give below an example of a nonnormal distribution where the quotient has the Cauchy distribution. This example, of course, does not rule out the possibility of characterizing a wider class of distribution laws by this property of the quotient.

EXAMPLE: Let $x$ and $y$ be two independently and identically distributed random variables, each having an absolutely continuous distribution function symmetrical about zero. Let the probability density function of $x$ be given by

$$f(x) = \frac{\sqrt{2}}{\pi} \cdot \frac{1}{1 + x^4} \qquad (-\infty < x < +\infty). \tag{1}$$

We introduce the polar transformation $x = r \cos \theta$, $y = r \sin \theta$ and can easily deduce that the joint probability density function of $r$ and $\theta$ has the form

$$c\,\frac{r}{(1+r^4\cos^4\theta)(1+r^4\sin^4\theta)} \qquad (0<r<\infty,\ 0<\theta<\pi) \tag{2}$$

where $c$ is a constant.

Now, in order to find the distribution of $\theta$, we have to integrate (2) with respect to $r$ over the range $(0, \infty)$.

Making the substitution $r^2 = u$, the probability density function of $\theta$ is given by

$$\frac{c}{2}\int_0^\infty \frac{du}{(1+u^2\cos^4\theta)(1+u^2\sin^4\theta)}. \tag{3}$$

From (3) it can be easily verified that $\theta$ has a uniform distribution in the interval $(0, \pi)$.

Finally, substituting $x/y = \cot\theta$ in the distribution of $\theta$, it is proved that the quotient $x/y$ follows the Cauchy distribution.

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## *SPIN, STATISTICS, AND THE TCP THEOREM*

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The recent experimental discoveries relating to space parity and charge symmetry in the so-called weak interactions have emphasized the need for a clearer recognition of the role played by the continuous Lorentz subgroup of proper, orthochronous Lorentz transformations. This refers, in particular, to some work of the author,[1] in which the general requirement of Lorentz invariance for the quantum theory of fields is used to deduce the connection between the spin and the statistics of particles, and to a converse statement (now known as the "TCP theorem") in which the acceptance of the spin-statistics connection implies invariance under a combined space, time, and charge-reflection operation.[2] It is the intention of this note to make explicit the fact that the general dynamical structure of the quantum theory of fields, together with the specific assumption of invariance under the proper orthochronous Lorentz subgroup, and the existence of a lowest-energy state (the vacuum) for any physically realizable system, implies both the connection between the spin and statistics of particles and the TCP theorem.

The dynamical principle of the theory of fields, governing the development of the system between two space-like surfaces, is the differential action principle,

$$\delta\langle\sigma_1|\,\sigma_2\rangle = \langle\sigma_1|\,\delta\Big\{i\int_{\sigma_2}^{\sigma_1}(dx)\mathfrak{L}[\chi]\Big\}\,|\,\sigma_2\rangle,$$

where

$$\mathfrak{L}[\chi] = {}^1\!/_4\,(\chi A^\mu\partial_\mu\chi - \partial_\mu\chi A^\mu\chi) - \mathfrak{IC}(\chi)$$

is the Lagrange function, a Hermitian operator function of the multicomponent Hermitian field, $\chi(x)$. Here $x$ refers to the space-time co-ordinates $x^0 = -x_0 = t$, $x_k = x^k$, and $k = 1, 2, 3$. The finite dimensional matrices $A^\mu$ are skew-Hermitian, and their complete reduction into the two classes of real antisymmetrical matrices and imaginary symmetrical matrices expresses the separation of the total field $\chi$ into the two fundamental classes of B(ose)-E(instein) fields $\phi$, and F(ermi)-D(irac) fields $\psi$. Corresponding to the symmetry of the relevant parts of the matrices $A^\mu$, the B-E fields must be essentially commutative, and the F-D fields anticommutative, in order that the first kinematical term of the Lagrange function be a non-trivial operator (this is a simplification of the complete discussion), and, similarly, the structure of $\mathfrak{H}$ must involve the $\phi$ and $\psi$ fields in a respectively, symmetrical and antisymmetrical manner. The development of the dynamical theory also shows that $\mathfrak{H}$ and hence $\mathfrak{L}$ are even functions of the F-D fields.

The Hermitian requirement on $\mathfrak{L}$ can now be given the following expression. On forming the Hermitian adjoint, the sense of multiplication of all the Hermitian operators $\chi$ is reversed ($^T$), and the sign of $i$ is changed wherever it appears explicitly ($^*$). But the Lagrange function is a symmetrical function of $\phi$ and an even antisymmetrical function of $\psi$, from which we conclude that

$$\begin{aligned} \mathfrak{L}[\phi, \psi] &= \mathfrak{L}^{T*}[\phi, \psi] \\ &= \mathfrak{L}^*[\phi, i\psi], \end{aligned}$$

the introduction of $i$ in the latter form being a convenient way of expressing the sign reversal of all terms in $\mathfrak{L}$ that contain an odd number of pairs of F-D field components. The use of complex numbers in quantum mechanics requires that with each abstract symbol there be associated a symbol in a complex conjugate algebra. On proceeding to this complex conjugate description, the form of the action principle is altered as follows:

$$^*{:}\delta\langle\sigma_1|\,\sigma_2\rangle = \langle\sigma_1|\,\delta\Big\{-i\int_{\sigma_2}^{\sigma_1}(dx)\mathfrak{L}[\phi, i\psi]\Big\}\,|\,\sigma_2\rangle,$$

in which all vectors and operators refer to the complex conjugate algebra.

The finite multiplicity of the Hermitian field $\chi$ pertains in part to space-time properties (spin) and in part to internal properties (charge, in a general sense). The spin degrees of freedom are made explicit in the transformation that accompanies infinitesimal Lorentz transformations, the generators of the proper, orthochronous Lorentz group. Thus the infinitesimal rotation,

$$\begin{aligned} \bar{x}^\mu &= x^\mu + \epsilon^\mu{}_\nu x^\nu \\ \epsilon_{\mu\nu} &= -\epsilon_{\nu\mu} \end{aligned}$$

induces

$$\bar{\chi}(\bar{x}) = [1 + i\,{}^1/_2\,\epsilon^{\mu\nu}S_{\mu\nu}]\chi(x),$$

where the imaginary matrices $S_{\mu\nu}$ are a finite-dimensional matrix realization of the four-dimensional angular momentum operators $J_{\mu\nu}$. But, unlike these operators, the matrices $S_{\mu\nu}$ are not Hermitian, for the Lorentz group possesses no finite-dimensional unitary matrix representation. The finite matrices refer rather to the Euclidean rotation group produced by the introduction of the imaginary time

$x_4 = ix^0$, and it is the matrices $S_{\mu\nu}$, $\mu\nu = 1, \ldots, 4$, $S_{k4} = iS_{0k}$, that are, or can be chosen, as Hermitian matrices. With the latter choice the matrices $S_{kl}$ are imaginary and antisymmetrical, while the $S_{k4}$ are real and symmetrical. Now, while the complete Lorentz group contains four pieces, corresponding to the distinction between proper and improper transformations and of past and future, the Euclidean rotation group distinguishes only between proper and improper transformations. Accordingly, if the Lagrange function is invariant in form under infinitesimal Lorentz transformations, it is invariant under infinitesimal Euclidean rotations, and, by a repetition of such infinitesimal rotations, the following *proper* transformation can be effected:

$$\bar{x}^\mu = -x^\mu.$$

The corresponding field transformation is

$$\bar{\chi}(\bar{x}) = R_{st}\chi(x),$$

where

$$R_{st} = e^{\pi i S_{12}} e^{\pi i S_{34}}$$

or any equivalent form that regards the co-ordinate transformation as a rotation through the angle $\pi$ in two perpendicular planes. The existence of the matrix $R_{st}$ is assured only for the bounded finite-dimensional spin representations, but this suffices for the purposes of physics.

The above transformation maintains the algebraic structure of the Lagrange function, but it does not necessarily preserve the reality properties that accompany the Lorentz group. Indeed, since $S_{34}$ is a real matrix,

$$R_{st}^* = e^{-2\pi i S_{34}} R_{st} = (-1)^{2S} R_{st},$$

in which we have recognized that the eigenvalue spectrum of $S_{34}$, which is the same as that of $S_{12}$, for example, differs by integers from the spin quantum number $S$. Accordingly, $R_{st}$ is real if $S$ is integral, but $R_{st}$ is imaginary for half-integral $S$. The formal transformation of space-time reflection thus maintains the Hermitian nature of fields that are associated with integer spin, but converts Hermitian half-integral spin fields into skew-Hermitian fields.[3] This is indicated in the new form assumed by the action principle,

$$R_{st}{:}\delta\langle\sigma_1|\,\sigma_2\rangle = \langle\sigma_1|\,\delta\Big\{-i\int_{\sigma_2}^{\sigma_1}(dx)\mathfrak{L}[\chi_{\text{int}}, i\chi_{1/2\,\text{int}}]\Big\}\,|\,\sigma_2\rangle,$$

where the sign change of the four-dimensional integral stems from the opposite time order in which the two spacelike surfaces are placed, in the new co-ordinate system.

The combination of the two operations—of complex conjugation and space-time co-ordinate reflection—now produces the action principle form

$${}^*R_{st}{:}\delta\langle\sigma_1|\,\sigma_2\rangle = \langle\sigma_1|\,\delta\Big\{i\int_{\sigma_2}^{\sigma_1}(dx)\mathfrak{L}[\phi_{\text{int}}, i\phi_{1/2\,\text{int}}, i\psi_{\text{int}}, \psi_{1/2\,\text{int}}]\Big\}\,|\,\sigma_2\rangle.$$

Thus the initial structure of the action principle is regained, and, if the system is described by the Lagrange function

$$\mathfrak{L}[\phi_{\text{int}}, \phi_{1/2\,\text{int}}, \psi_{\text{int}}, \psi_{1/2\,\text{int}}],$$

it is equally well described by the Lagrange function,

$$\mathfrak{L}[\phi_{\text{int}}, i\phi_{1/2\,\text{int}}, i\psi_{\text{int}}, \psi_{1/2\,\text{int}}].$$

In particular, the kinematical terms for the field types $\phi_{1/2\,\text{int}}$, $\psi_{\text{int}}$ appear with opposite signs in the two Lagrange functions. It is at this point that the physical requirement of a lowest-energy state can be introduced. The energy operator contains that part of the kinematical term which involves spatial derivatives, and it is this contribution that dominates the high-energy limit of the spectrum where arbitrarily rapidly varying fields occur. Indeed, it is basic to the (over)-idealized measurement theory which underlies the quantum theory of fields that the couplings between fields are ineffective in comparison with the kinematical properties of the individual fields over infinitesimal space-time regions, or, equivalently, in the indefinitely elevated portions of the energy spectrum. Now, if the first Lagrange function carries the implication that the highly excited part of the energy spectrum associated with the fields $\phi_{1/2\,\text{int}}$, $\psi_{\text{int}}$ extends to $+\infty$, the second Lagrange function will state that it also extends to $-\infty$. Therefore, no vacuum state can be defined. The resulting inference that only fields of the type $\phi_{\text{int}}$, $\psi_{1/2\,\text{int}}$ exist in nature is the connection between spin and statistics. And the conclusion, for the latter fields, that the transformations of space-time reflection and complex conjugation leave invariant the form of the action principle is the TCP theorem.

One point still requires attention, however. We have spoken of complex conjugation but not of charge reflection, and, indeed, no explicit reference to the internal properties of the system has appeared in our discussion. This is attributable to the use of Hermitian fields, which, individually or in the multiplicity necessary to incorporate spin, do not describe internal characteristics but rather accomplish this through an additional degeneracy. Any internal property in the nature of a charge, be it nucleonic charge, hyperonic charge, electrical charge, or leptonic charge, arises from an internal symmetry transformation,

$$\bar{\chi} = (1 + i\delta\lambda q)\chi,$$

where $\delta\lambda$ is an infinitesimal real parameter, and the matrix $q$ is antisymmetrical and imaginary. Unlike the Lorentz transformations, the group of continuous internal symmetry transformations is a compact group defined in a Euclidean space. Associated with the matrix $q$ is the Hermitian operator $Q$ of total charge, which is the generator of the infinitesimal unitary transformation that is equivalent to the $\lambda$ transformation of the field. This is expressed by the commutation relation,

$$[\chi, Q] = q\chi.$$

As an antisymmetrical imaginary matrix, $q$ possesses real eigenvalues which are symmetrically distributed about zero. For the fundamental situation of $q' = \pm 1$, the explicit construction of the fields, $\chi_{q'}$, that obey

$$[\chi_{q'}, Q] = q'\chi_{q'}$$

is given by

$$\chi_{q'} = {}^1/_2 (1 + q'q)\chi.$$

These fields, labeled by charge eigenvalues, are non-Hermitian,

$$\chi_{q'}\dagger = \chi_{-q'}.$$

In a similar way, the transition to the complex conjugate algebra replaces $\chi_{q'}$ with $\chi_{-q'}$ and $Q$ with $-Q$. Hence the combination of the two operations—of complex conjugation and the Hermitian adjoint—which is transposition, leaves $\chi_{q'}$ unaffected while changing $Q$ into $-Q$ (this accounts for the emphasis on transposition in Paper I before the utility of Hermitian fields was appreciated). These references to complex conjugation must be understood to include the space-time co-ordinate reflection needed to preserve the structure of the action principle and thereby of the field operator commutation properties.

The explicit indication of charge reflection appears, not in the form of the action principle, but in the detailed correspondence of states that are related through the combined operation of space-time reflection and complex conjugation. If we consider a state labeled by one or more charge values $Q'$, the corresponding state possesses the charge values $-Q'$. Charge is analogous to angular momentum in this respect. The three-dimensional angular-momentum operators are represented by imaginary matrices (and differential operators). Hence, if a state possesses a definite magnetic quantum number, say $J_3'$, the corresponding state, after complex conjugation and space-time reflection, exhibits the magnetic quantum number $-J_3'$. Of course, the tensor character of the angular-momentum operator is also relevant; for, in contrast, the vector of energy and momentum, $P_\mu$, appears unchanged in form after complex conjugation and space-time reflection. We are thus assured that if a state exists with the properties $P_\mu'$, $Q'$, $J_3'$, so also does the state characterized by $P_\mu'$, $-Q'$, $-J_3'$. This is the ultimate basis for the conviction that every particle which displays one or more charge attributes possesses an antiparticle of identical mass.

The automatic appearance of charge reflection in response to complex conjugation provokes a speculation on the fundamental significance of the use of complex numbers in quantum mechanics. The mathematical machinery of quantum mechanics is a symbolic expression of the laws of atomic measurement, abstracted from the specific properties of individual techniques of measurement.[4] In particular, the space-time manifold that is the background of any quantum-mechanical description is an idealization of the function of a measurement apparatus to define a macroscopic frame of reference. Now one may ask whether there is any other general property of matter in bulk that may be significant in its role as the substance of the instrument of measurement. It is tempting to cite here the evident requirement of permanence—if the measurement device is to fulfill its final function of providing a record of the results obtained—which would appear to limit its atomic constituents to a stable configuration of particles or of antiparticles. And, from another direction, all measurement involves thermodynamically irreversible changes in the measurement instrument, which could hardly be detected over the vastly more drastic annihilation processes of matter and antimatter. Thus, if we disregard all specific details of the substance composing the apparatus of measurement, we are still left with the twofold choice of matter or antimatter.

Is it possible that the symbol of this aspect of the measurement instrument is $\pm i$? The mathematical operation of complex conjugation would then be given the physical interpretation of the change in description that accompanies the substitution of an antimatter apparatus for a matter apparatus or conversely, and it becomes comprehensible that such a description change requires the interchange of particles and antiparticles if the intrinsic relation between system and measurement instrument is not to be altered. Quantum field theory can be reconstructed on this basis, but we shall defer the detailed discussion for another occasion.

[1] J. Schwinger, *Phys. Rev.*, **82**, 914, 1951; *ibid.*, **91**, 713, 1953, referred to as Paper I and II, respectively.

[2] A commentary on Paper I and related work of other authors will be found in the article of W. Pauli in *Niels Bohr and the Development of Physics* (New York: McGraw-Hill Book Co., 1955).

[3] The whole discussion differs only in emphasis from that of Paper II.

[4] The possibility thus suggested of a direct physical motivation of the otherwise remote mathematical entities of quantum mechanics has been under development since 1951 and at several stages has been published in various (inaccessible) places. Perhaps the most complete account appeared in *Lectures at the Summer School of Theoretical Physics, Les Houches, France, 1955.*

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